CHEK2 gene mutations, CYP1B1 gene 355T/T polymorphism, and HPV infection approached as urinary bladder cancer predisposing factors

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KEY WORDS

bladder cancer ◗ factors ◗ CHEK2 mutations ◗ CYP1B1 polymorphism ◗ predisposition ◗ HPV infection

ABSTRACT

Introduction. CHEK2 and CYP1B1 are among the genes of which mutations may increase the risk of cancer incidence. A multi-organ predisposition of these genes to induce neoplasm development has been reported. Human papilloma virus (HPV) can participate as an environmental factor in neoplastic transformation towards urinary bladder carcinoma. Scarce literature data suggest that HPV infections are fairly common and may be a risk factor for urinary bladder carcinoma. However, no eventual conclusions have been presented. For this reason, a study of a large patient population and of health controls seems fully justified.

Material and methods. Fifty (50) patients with diagnosed urinary bladder cancer were qualified to the study. The control group included 50 healthy subjects with no diagnosed neoplastic changes. Mutations/polymorphisms of CHEK2 and CYP1B1 were identified by the PCR in DNA isolated directly from the tumor and from peripheral blood. The ELISA test was use for the studies of 37 HPV genotypes in DNA, isolated from the tumor.

Results. CHEK2 gene mutations were identified in five cases (5/50) and 355T/T polymorphism of CYP1B1 gene was observed in nine patients (9/50). HPV was identified in seventeen cases (17/50).

Conclusions. The prevalence of CHEK2 mutations in the study group was similar to literature data while the prevalence of the A119S variant of the CYP1B1 gene was higher than that reported in the literature. The presence of HPV DNA was detected with the incidence rate, close to that in reports of other authors.

INTRODUCTION

The formation, development, and progression of neoplasms constitute a complex process accompanied by a series of various genetic abnormalities. It is assumed that a number of genes participating in the control processes of the cell life cycle, its growth, tissue specialization, and death may be involved in the transformation of a normal cell into a neoplastic cell. The knowledge and understanding of the status of the genes, which participate in various cellular „pathways", can be fairly significant in the prognostic evaluation of patients with urinary bladder cancer as well as introduce important clinical implications directly transposing onto therapy schemes.

The development of molecular biology, which can be observed during the recent years, has contributed to better consolidation and strengthening of the knowledge concerning the molecular basis of carcinogenesis that has, in consequence, given us better understand of the genetic causes leading to urinary bladder cancer development.

Urinary bladder cancer in Poland has been the fourth malignant neoplasm in prevalence ranking in males (6.1%) and the seventeenth in females (1.6%), while deaths of males in result of urinary bladder cancer are the fifth in the ranking of death caused because of neoplastic diseases [1]. Urinary bladder cancer, similarly as other malignant neoplasms, is a serious medical, ethical, and social problem of today's medicine. Following the basic rules of medical practice, the problems associated with neoplastic diseases should be evaluated in two planes – prevention and implementation of prophylactic protocols and early diagnostics. Broadly understood diagnostics of neoplastic disease includes detection, proper identification of neoplastic disease, prognosis, and the selection of an optimal therapeutic protocol. Prevention is possible in the preclinical phase of neoplasm development and is addressed to patients from high risk groups. Therefore, it is of particular importance to select such groups, not only on the basis of environmental cancerogenic factors (occupational and extra-occupational), but also taking into consideration the genetic factors that may support neoplastic progression. Determination of the frequency of CHEK2 gene mutations, CYP1B1 polymorphism, and of other genetic factors in tumor cells is the first step to enable statistical evaluation of the extent of individual risk for disease and to evaluate genetic predisposition to urinary bladder cancer in the process of its complex prophylactics.

In cells of urinary bladder cancer, a number of abnormalities at the molecular level as well as chromosomal aberrations have been described. Loss of heterozygosity (LOH) of chromosome 9 is observed in the majority of superficial tumors. Deletions of chromosome 9 occur in more than 50% of all TCC tumors. LOH of 9p21 is present both in papillary urothelial neoplasms of low malignancy (Ta) as well as in the invasive urinary bladder cancer, while a reduced number of 9p21 copies, either with or without LOH, is observed in approximately 45% of tumors [2]. Studies of molecular genetic factors in urinary bladder cancer cells also indicate the occurrence of changes in the following regions of chromosomes: 3p21-25, 11p15, 13q14, 17p11-13, and 18q21, which encode suppressor genes TP53 and RB1, among others [3]. Here, genetic factors are responsible both for single- and multiple-gene predisposition as well as

for "high" and "moderate" predisposition. Thus, there are "highly" predisposing genes or genes that moderately increase the risk for malignant neoplasm development. Moreover, the concurrence and synergistic activity of "weak" mutations/polymorphisms in many genes and the effects of environmental factors may decisively increase the risk for cancer occurrence [4].

The genes moderately increasing the risk for malignant neoplasm formation, include: CHEK2 and CYP1B1.

CHEK2 gene is localized on the long arm of chromosome 22q12.1 and codes CHEK2 protein – protein kinase, which is activated by DNA lesions. Four mutations of the gene are found in the Polish population: IVS2 + 1G>A, 1100delC, del5395, and I157T. Mutations of the CHEK2 gene predispose, via multi-organ effects, to neoplasm development. IVS2 + 1G>A, 1100delC, and del5395 changes increase the risk of thyroid, breast, and prostate cancer. The I157T variant is associated with an increased risk of breast, colorectal, renal, prostate, thyroid cancer, and ovarian cancer of the low degree of morphological malignant potential [4]. Studies, performed in patients with advanced urinary bladder cancer, demonstrated either a decreased number or a total loss of the CHEK2 protein [5].

CYP1B1 gene is localized on chromosome 2p22-21 and contains 3 exons. It encodes CYP1B1 protein, which is an extrahepatic protein – P450. There are contradictory reports about its expression in healthy tissues, while high expression levels of CYP1B1 protein have been demonstrated in certain types of neoplasms: breast, colon, lung, esophagus, skin, brain, small intestine, and uterine cancer [6]. Some authors describe CYP1B1 protein presence in prostate cancer tissues in 75% of studied cases and in urinary bladder tumors in 100% of studied cases [7].

According to the latest literature reports, one of the most frequent factors taking part in carcinogenesis in men is the human papilloma virus (HPV). Genotypes of HPV identified in invasive types of neoplasms have been identical with the types that experimentally demonstrated the ability to transform human cells, thus being regarded as high risk HPV types and include: type 16, 18, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82, and 83 [8]. Some authors present data according to which HPV has been found in 50% of the studied cases of urinary bladder cancer [9], while others approach this correlation as rather doubtful or impossible [10].

Despite the dynamic development of molecular biology and advanced knowledge on carcinogenesis, researchers have been struggling with problems concerning the interpretation of obtained study results and of their diagnostic and/or prognostic value. For the above mentioned reasons, studies performed on large patient populations vs. health controls seem mostly justified.

MATERIAL AND METHODS

Selected mutations of CHEK2 (I157T, IVS2 + 1G>A, 1100delC, and del5395), polymorphism of CYP1B1(355 T/T), and of HPV virus were identified in DNA isolated from materials collected from the subjects of both the study and the control groups according to the below presented algorithm. Altogether the studies involved 100 subjects: 50 with diagnosed urinary bladder carcinoma and 50 healthy controls with no diagnosed neoplastic changes or other urological diseases.

Patients

The study material included DNA isolated from tumor cells and from peripheral blood. In order to determine whether the identified mutations/polymorphism of CHEK2 and CYP1B1 were confined to neoplastic cells or if they occur constitutively, they were detected in DNA of tumor cells. Then, in the patients with detected mutation/polymorphism in tumor cells, the constitutive character of the observed mutation/polymorphism was confirmed by an analogous evaluation of DNA in peripheral blood.

In the study group, HPV DNA was detected in tumor cells.

Control group

The study material used for the identification of mutations/polymorphisms of the analysed genes included DNA isolated from peripheral blood only. The performed studies of CHEK2 and CYP1B1 mutations/polymorphisms in the DNA of tumor cells from 50 patients with diagnosed urinary bladder carcinoma demonstrated a constitutive character of all the identified mutations/polymorphisms, which was present in all the tissues. Therefore, regarding the control group, they were not detected in the DNA isolated from urinary sediment cells, but in the DNA from peripheral blood.

In the control group, HPV DNA was identified in urinary sediment cells.

DNA isolation

1. DNA from tumor cells and urine sediment's cells was isolated by the method with ion-replaceable membranes (a SHERLOCK AX kit of the A&A Biotechnology Company)
2. DNA from whole peripheral blood was isolated by automated methods, using glass magnetic balls [11]

DNA quality was evaluated after electrophoresis in 1% agarose gel with an addition of ethidine bromide.

Mutation detection

The selected 3 mutations of the CHEK2 (IVS2 + 1G>A, 1100delC, and I157T) gene were detected by the multiplex PCR using genetic tests designed at the Department of Genetics and Pathomorphology, Pomeranian Medical University in Szczecin and published elsewhere before [12]. The fourth mutation of CHEK2 (del5395) was detected using PCR [13], while the 355T/T change of the CYP1B1 gene were detected by the RFLP-PCR method [14].

HPV detection

HPV DNA was detected by means of the LINEAR ARRAY Human Papillomavirus GENOTYPING Test (Roche), which enables simultaneous PCR of the target HPV DNA and of β-globin DNA as an internal cellular control. The test employs primer pairs to define the sequences of nucleotides within the polymorphic L1 region of HPV genome. A pool of HPV primers in the test includes 37 pathogenic genotypes: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, and 84. PCR are submitted to amplification with oligonucleotide probes, followed by detection with color reaction [15].

RESULTS

Detection of four (4) CHEK2 mutations, polymorphism 355 T/T of CYP1B1, and identification of HPV was performed in 50 patients and 50 controls. Three (3) CHEK2 (IVS2 + 1G>A, 1100delC, and I157T) gene mutations were detected in 5 of the 50 (10%) patients with urinary bladder carcinoma, including 3 cases of G1 tumor, 1 case of G2 tumor, and 1 case of PUNLMP. CYP1B1 gene 355T/T polymorphism was found in 9 of the 50 (18%) patients with urinary bladder carcinoma, including 7 cases of G1 tumor, 1 case of G3 tumor, and 1 case of PUNLMP. In all the mutation-positive cases, the change occurred constitutively – being present both in tumor cells and in peripheral blood cells. In turn, in the control group, only the CHEK2- I157T mutation was found in 2 of the 50 (4%) healthy subjects. No other CHEK2 mutations, i.e., IVS2 + 1G>A, 1100delC, observed in the patients with urinary bladder carcinoma were

Table 1. Characteristics of the study population and results of CHEK2, CYP1B1 mutations/polymorphism, and HPV infection.

No.	Sex	Age	Staging	Grading	CHEK2	CYP1B1	HPV	No.	Sex	Age	Staging	Grading	CHEK2	CYP1B1	HPV
1	M	53	Ta	G1	+	T/T	–	26	M	54	Ta	G1	–	G/G	+
2	K	72	PUNLMP		–	G/G	–	27	M	63	PUNLMP		–	T/T	–
3	M	66	T2	G2	–	G/G	+	28	M	70	Ta	G1	–	G/G	–
4	M	52	T1	G1	–	G/G	–	29	M	82	T2	G1	–	G/G	–
5	M	66	T2	G2	–	G/G	+	30	M	67	PUNLMP		–	G/T	–
6	M	73	T1	G2	+	G/G	–	31	M	76	Ta	G1	–	G/T	–
7	M	71	Ta	G1	–	G/T	–	32	M	55	T2	G1	–	T/T	–
8	M	57	Ta	G1	+	G/T	–	33	M	71	T1	G1	–	G/T	–
9	M	66	Ta	G1	–	T/T	–	34	M	54	PUNLMP		–	G/T	–
10	M	47	PUNLMP		+	G/G	–	35	M	77	T2	G3	–	G/T	+
11	M	74	Ta	G2	–	G/T	+	36	M	84	T1	G3	–	G/T	+
12	M	72	T2	G2	–	G/T	–	37	M	83	T2G1	G1	–	G/T	–
13	M	86	T1	G2	–	G/T	+	38	M	72	T1	G1	–	G/T	–
14	M	74	Ta	G1	–	T/T	–	39	M	55	Ta	G1	–	G/G	–
15	M	32	PUNLMP		–	G/T	–	40	M	67	Ta	G1	–	T/T	–
16	M	70	T2	G3	–	G/G	+	41	M	71	T1	G1	+	G/G	–
17	M	65	T2	G3	–	G/T	+	42	M	70	T1	G1	–	T/T	–
18	M	66	Ta	G1	–	T/T	–	43	M	83	T1	G3	–	G/G	+
19	M	84	T1	G2	–	G/G	–	44	M	52	Ta	G1	–	G/G	–
20	M	76	T2	G3	–	T/T	+	45	K	79	Ta	G1	–	G/G	–
21	M	53	T1	G3	–	G/T	–	46	K	88	T2	G3	–	G/T	+
22	M	84	PUNLMP		–	G/T	–	47	M	57	T1	G1	–	G/T	–
23	K	61	T2	G3	–	G/G	+	48	M	73	Ta	G1	–	G/G	+
24	M	60	Ta	G2	–	G/T	–	49	M	52	Ta	G3	–	G/G	+
25	K	76	T2	G2	–	G/T	+	50	M	73	T1	G3	–	G/G	+

*median of age – 70

Table 2. Prevalence of mutations/polymorphism of CHEK2 and CYP1B1 and detection of DNA of HPV in cases and controls.

	Material	Detected mutation/polymorphism					DNA of HPV
		CHEK2-I157T	CHEK2-IVS2 + 1G>A	CHEK2-1100delC	CHEK2-del5395	355 T/T	
Studied group	Tumour	3/50	1/50	1/50	0/50	9/50	17/50
	Blood	3/3	1/1	1/1	–	9/9	–
Control group	Blood	2/50	0/50	0/50	0/50	5/50	5/50*

*detection in DNA isolated from urine samples

found in the population of healthy controls. Neither in the study group nor in the control group was the CHEK2-del5395 mutation observed. The 355T/T polymorphism of CYP1B1 gene was found in 5 of the 50 (10%) healthy subjects. See Tables 1 and 2 and Figures 1, 2, 3, and 4 for detailed results of the studies.

The pathogenic HPV virus was identified in 17 of the 50 (34%) patients with urinary bladder carcinoma and in 5 of the 50 (10%) healthy controls.

DISCUSSION

In our patients with diagnosed urinary bladder cancer of various grading, slight statistical differences were found in the prevalence of CHEK2 and CYP1B1 mutations/polymorphisms between the study group and the control group. The prevalence of CHEK2 mutations were at the level of 10% (5/50) and at the level of 4% (2/50), in the study group and in the control group, respectively. The missense mutation (I157T) prevalence rate was 6% (3/50), while the IVS2 + 1G>A and 1100delC mutations occurred with a total prevalence rate of 4% (2/50). In the controls, CHEK2 gene mutations were found in two cases (2/50) with a total prevalence of 4% and variant missense. The studies of IVS2 + 1G>A, 1100delC, and I157T mutations of the CHEK2 gene performed in the Polish population determined the prevalence rate of CHEK2-IVS2 + 1G>A and 1100delC at 0.7% in the control group and I157 change at 4.8%. In our studies, a similar prevalence rate of I157T mutations was observed in the control group – 4%, while mutation results in a shortened protein (IVS2 + 1G>A, 1100delC, and del5395) did not occur in our control group. Following literature data, CHEK2 gene mutations are slightly more frequent in patients with malignant

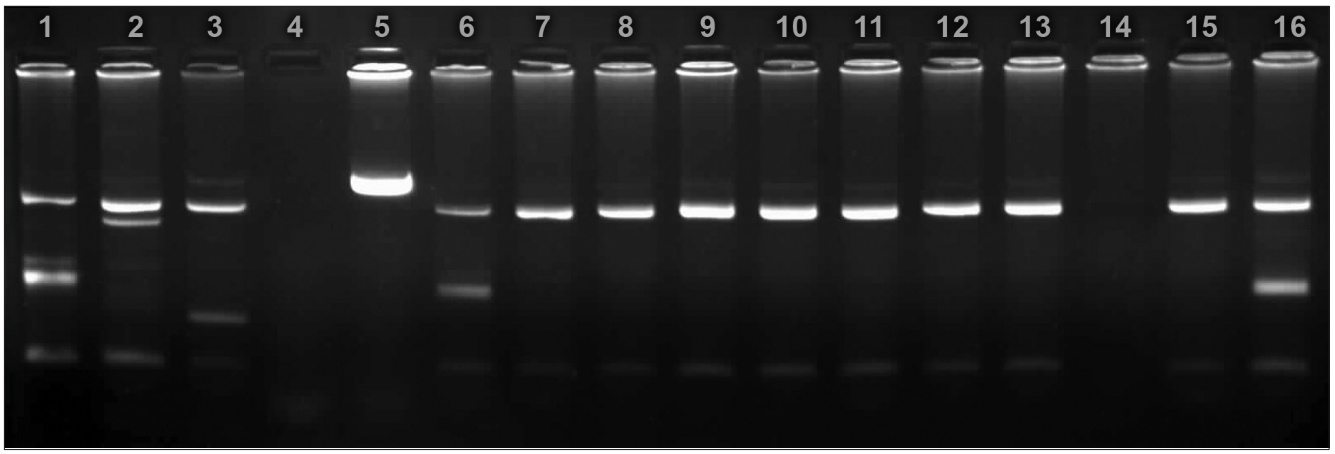

Fig. 1. Results of CHEK2 analysis – multiplex PCR: 1 – I157T positive control; 2 – IVS2 + 1G> A positive control; 3 – 1100delC positive control; 4, 14 – negative "blind" controls; 5 – PCR product; 7-13, 15 – studied samples – negative; 6, 16 – studied sample – positive I157T.

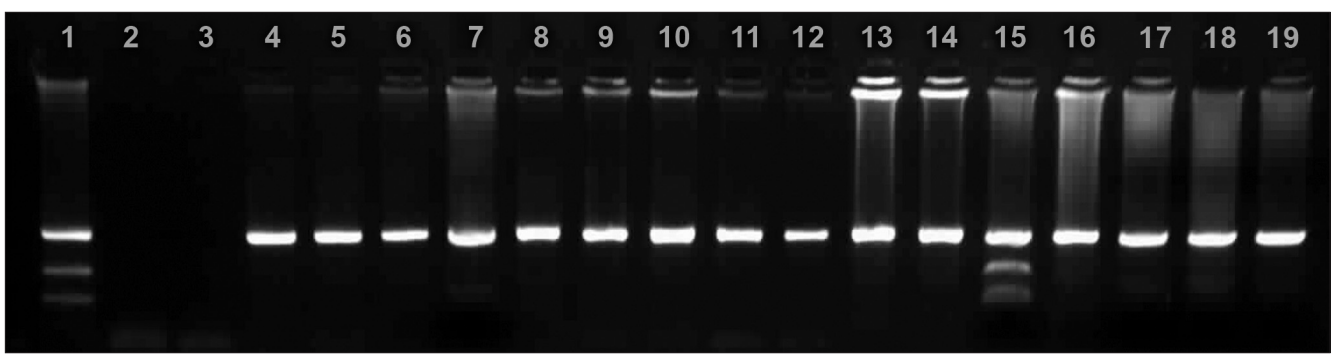

Fig. 2. Results of CHEK2 analysis – IVS2 + 1G>A: 2,3 – negative "blind" controls; 2 – IVS2 + 1G>A positive control; 4-14, 16-19 – studied samples – negative; 15 – studied sample – positive.

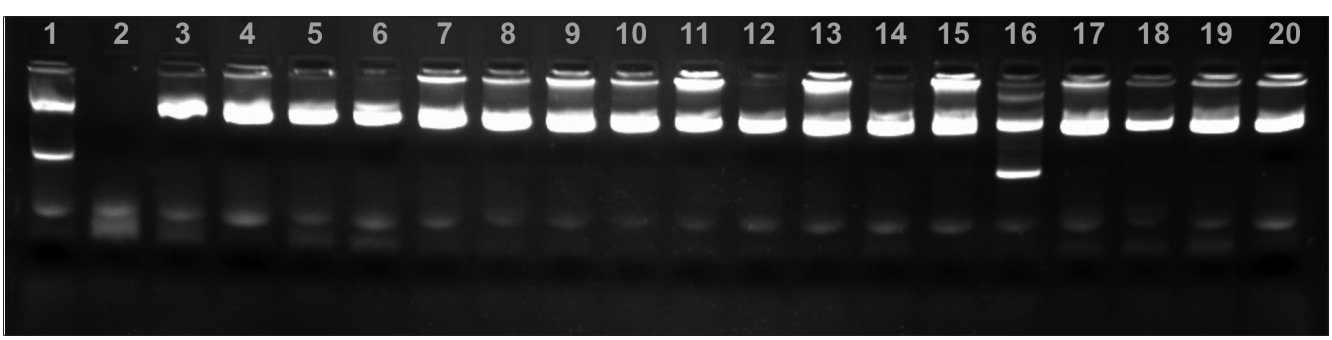

Fig. 3. Results of CHEK2 analysis – 1100delC: 1 – 1100delC positive control; 2 – negative "blind" control; 3-15, 17-20 – studied samples – negative; 16 – studied sample – positive.

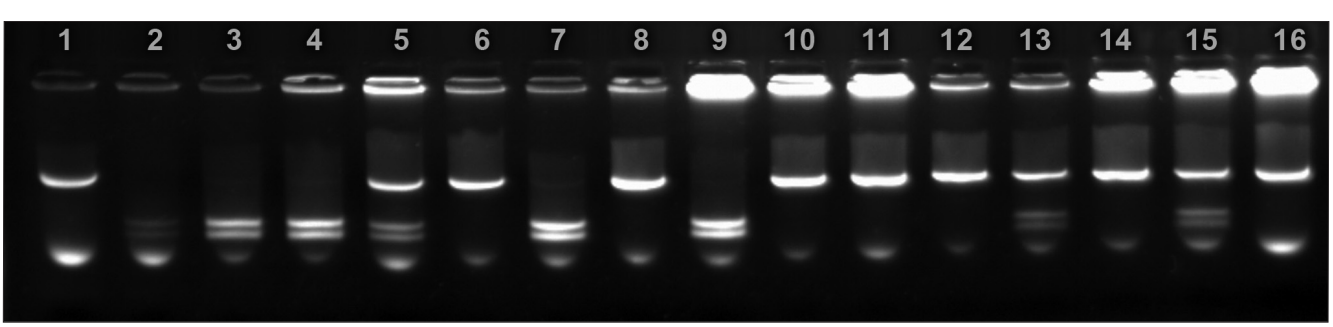

Fig. 4. Results of 355T/T CYP1B1 analysis: 1,6,8,10-12,14,16 – studied sample, homozygotes G/G; 4 – positive control, homozygote T/T; 5,13,15 – studied sample, heterozygote G/T; 2-3, 7,9 – studied sample – homozygotes T/T.

neoplasms. IVS2 + 1G>A and 1100delC mutations of the CHEK2 gene in patients with thyroid neoplasms were at the prevalence level of 3.5%, gastric – 2.1%, prostate – 1.6%, and breast – 1.6%. The CHEK2 protein shortening mutations in our studies, in the group of patients with urinary bladder carcinoma, amounted to 4%, which demonstrates a slightly higher prevalence, however it

may be connected with the small number subjects in the study group. In turn, the prevalence of the I157T mutation of the CHEK2 gene demonstrated the following values in the groups of patients with neoplasms of the following organs: the prostate – 7.8%, the thyroid – 8.7%, the kidneys – 9.8%, the colon – 9.3%, and the breast – 6.7% [16]. The prevalence of a missense mutation in the studies of our patients with urinary bladder carcinoma was 6%, being similar to the prevalence in the group of patients with breast cancer, as reported in the referenced data.

Scarce studies of the CHEK2 protein in cells of urinary bladder cancer indicate a positive result of immunohistochemical staining with the application of antibodies against the active forms of the CHEK2 protein, in the prevailing majority of non-infiltrating Ta tumors (following TNM classification: non invasive papillary carcinomas) and of tumors in T1 stages (following TNM classification: tumors infiltrating the submucosal layer of the urinary bladder). Staining results were moderately lower in the invasive forms of urinary bladder cancer - T2-T4 [17]. Subsequent immunohistochemical studies of 58 cases in advanced (T2-T4) stages demonstrated either signal reduction or loss in neoplastic cells (in 6 tumors). After molecular studies were performed in all those cases, in one patient 1100delC mutation was detected in one allele and the occurrence of the above mentioned mutations was confirmed to terminal cells [5]. Other authors studied four mutations in the CHEK2 gene in patients with urinary bladder cancer in the Polish population and found out that their prevalence rate was 10.6% in that group, while achieving the level of 5.9% in the control group [18], which is conformable to our results: 10% in the study group and 4% in the control group, respectively.

Polymorphism of CYP1B1 (355T/T) occurred in 18% (9/50) of patients in the study group and its occurrence was constitutional in all the patients with somatically confirmed 355T/T polymorphism. In controls, the CYP1B1polymorphism occurred in five cases (5/50).

Literature data include reports of CYP1B1 protein expression studies in cells of urinary bladder cancer, using immunohistochemical methods. The application of monoclonal antibodies, specific for 437 and 451 amino acid sequences of CYP1B1 protein in tumor tissue of 22 patients from the transitional cell cancer (TCC) and in metaplastic and hyperplastic urothelium, enabled detection of protein expression in all the studied TCC cases (100%) and in the studied meta- and hyperplastic tissues [7]. In the studies of other authors an application of antibodies specific for 332 and 345 amino acid sequences of CYP1B1 protein confirmed with protein expression in patients with malignant neoplasms of the urinary bladder, brain, breast, colon, kidneys, stomach, testes, ovaries, skin, and lungs. CYP1B1 protein expression occurred in all the studied TCC cases [6]. Literature data inform about the prevalence rate of 355T/T polymorphism in the Polish population at 8.4% in the control group and at 11.4% in patients with breast cancer [19]. In our studies, we obtained a much higher prevalence of the above mentioned change, namely 10% and 18% in the control and study group, respectively. The fact of the small number of patients in our study and control group should be emphasized vs. the data in reports in the presented list of literature. It should be kept in mind that the differences in results of particular authors may have resulted from different numbers of patients in those groups.

The detection of oncogenic HPV types in the studied samples was at the level of 34% (17/50) and 10% (5/50) in the control samples. Literature data present studies of HPV DNA in paraffin sections of the urinary bladder [20, 21]. Barghi et al. performed a comparison of the prevalence of: 6, 11, 16, 18, 31, 33, and 35 virus types in 59 samples from patients with diagnosed TCC urinary bladder cancer and in a 20-subject control group. The detection rate of HPV in the study group was 35.6% (21/59), while in the control group only HPV DNA was detected in 1 case. Other authors applied the ISH (*in situ* hybridization) technique in order to detect HPV DNA in paraffin sections from 43 patients with urinary bladder cancer and obtained a result at the level of 39.5% (17/43). In our studies, we obtained a similar prevalence of HPV DNA in the study group, namely 34%. It is interesting that a pathogenic HPV virus was detected in all G3 tumors and not in any of G1 tumors. We assume then that HPV virus infection may be related to the clinical progression and differentiation of urinary bladder carcinoma. An analysis performed by one of the authors of the 5 reports concerning the correlations among the prevalence rates of urinary bladder carcinoma has demonstrated that in a total of 239 cases of the carcinoma, HPV DNA was detected in 61 patients, which makes the prevalence rate 25.5%, while in the control group with 52 cases HPV DNA's detection rate was 11.5%. The obtained OR of 2.6 indicates a potential relationship between the infection with an oncogenic virus type and urinary bladder carcinoma [22]. Some publications suggest a correlation of pRb protein inactivation by E7 protein of oncogenic HPV types with excessive cellular growth as well as an influence of E6 and E7 oncoproteins of HPV type 16 on the "delay" of chromosomes during mitosis that may lead to chromosomal instability, a process which is regarded to be an integral part of conversion of a normal cell into a neoplastic cell [20].

CONCLUSIONS

In the performed studies no significant differences were observed between the study group and the control group regarding the prevalence of the CHEK2 - I157T mutation. IVS2 + 1G>A and 1100delC mutations causing CHEK2 gene protein shortening occurred more frequently in the study group than in the control group, however, it could perhaps be attributable to the small number of patients in the study's evaluated groups. CHEK2 gene mutations occurred in the studied group with the total prevalence rate close to the prevalence rate reported by other authors [18], while CHEK2-IVS2 + 1G>A and 1100delC occurred with the prevalence rate significantly higher than the prevalence rate of the mutation in reports of other authors. The prevalence of I157T mutation in the control group is comparable with literature data. [16]. The 355T/T variant of CYP1B1 gene was detected with a prevalence rate significantly higher in the studied group than in the control group and slightly higher in the control group than provided by literature data [19], which may have been caused by the small number of patients in the studied groups. The presence of HPV DNA was detected with a prevalence rate close to that in reports of other authors [20, 21]. The presented results of the pilot studies are to be approached as a preliminary report, preceding a much broader study.

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