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Genetic contributions of MHC class I antigen processing and presentation pathway to bladder cancer risk and recurrence

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Human leukocyte antigen class I (HLA class I) antigen processing and presentation pathway (APP) defines anti-tumor immune response. ERAP, TAP, tapasin (TAPBP), and IFNγ modulate APP: control HLA class I expression in the tumor and the repertoire of presented tumor antigens. At the same time, vascular endothelial growth factor (VEGF) acts as an immunomodulator in the tumor microenvironment. The objective of the current study was to examine the association of single nucleotide polymorphisms (SNPs) in the ERAP1, ERAP2, TAP1, TAP2, TAPBP, IFNG genes with the corresponding mRNA expression in bladder cancer (BC) risk and recurrence after transurethral resection of BC. Moreover, we assessed the relationship between HLA class I and VEGF plasma levels and BC recurrence. We analyzed 9 SNPs in 124 BC patients using TaqMan genotyping and compared them with the data from 503 healthy individuals from the 1000 Genomes Project. In addition, we quantified the effects of SNPs on the corresponding mRNA expression in tumor and non-tumor adjacent tissue in 60 BC patients with primary and 30 with recurrent tumor by quantitative real-time PCR. Furthermore, the plasma HLA class I and VEGF levels were analyzed in BC patients and healthy controls by ELISA. IFNG (rs1861493) was associated with BC risk, TAPBP (rs3106189, rs2071888) with recurrence-free survival (RFS). Moreover, TAPBP mRNA expression was lower in tumors than in the adjacent tissue. The SNPs ERAP2 (rs251339) and TAP2 (rs241447, rs241448) variants affected mRNA expression in BC tissue. In tumor tissue, the high mRNA expression of ERAP1 was more common in BC patients with single tumors, ERAP2 in non-smokers, and TAP2 mRNA in recurrence. The lower HLA and higher VEGF plasma levels were observed in BC patients compared with healthy controls. We conclude that the genetic elements responsible for MHC class I APP may influence the BC risk, risk of recurrence, and RFS.

Key words: bladder cancer, risk, recurrence, MHC class I, antigen presentation pathway

Bladder cancer (BC) is estimated to be the most common malignant tumor among urological cancers worldwide [1]. Age, gender, environmental and occupational exposure to carcinogens, as well as chronic inflammation and dysfunction of the immune system, are essential factors contributing to the pathogenesis and risk of BC [2, 3].

Characterized by a high risk of metastasis, muscle-invasive bladder cancer (MIBC) is the most frequent cause of mortality among BC patients regardless of the treatment. High-grade non-muscle-invasive bladder cancers (NMIBCs) are characterized by frequent invasion and metastasis. Especially T1G3-early-stage and poorly-differentiated NMIBC has a high potential for recurrence and progression [4]. About 50–70% of patients with NMIBC suffer frequent relapses, and 10–20% of them progress to MIBC [5, 6]. The standard treatment procedure comprises transurethral resection of bladder tumor (TURBT) with or without immunotherapy, and a patient is followed up afterward [7]. Unfortunately, despite early detection and treatment, cancer relapses many times. The recurrence of the invasive tumor to muscle membrane after TURBT is the most common treatment failure.

Currently, the most important prognostic factors for BC include the number and size of tumors, tumor stage and grade, and the frequency of relapses. The European Organization for Research and Treatment of Cancer risk calculator is used to assess BC recurrence risk [8]. Unfortunately, none of the above tumor characteristics are reliable in predicting the course of the disease and recurrence risk in patients after TURBT. Therefore, it is essential to identify diagnostic biomarkers that predict the relapse of BC after TURBT. Sensitive and objective predictors identified by molecular methods

could be used to monitor patients undergoing therapeutic procedures and complementary routine cytological tests, thus contributing to more effective treatment options and improved survival of patients [9]. However, little is known about the genetic mechanism of BC recurrence.

The evasion of the immune response is considered a prerequisite for tumors occurrence and considered one of the hallmarks of cancer. The immune system recognizes and destroys tumor cells. Major histocompatibility complex class I (MHC class I) antigen processing and presentation pathway (APP) is essential in the presentation of tumor antigens to CD8+ T cells and thus plays a crucial role in tumor eradication [10, 11]. Alterations in the expression of major players in antigen processing affect MHC class I levels and significantly change the repertoire of peptides being presented. MHC class I APP is a complex process with many elements [12]. The main players include endoplasmic reticulum aminopeptidase associated with antigen processing (ERAP); peptide transporter associated with antigen processing (TAP); and tapasin, TAP-binding protein (TAPBP); which are induced by interferon-gamma (IFNG) [13–15]. Moreover, tumor recurrence depends on the process of angiogenesis through vascular endothelial growth factor (VEGF). VEGF is a significant factor in angiogenesis overexpressed in numerous malignancies, including BC [16, 17].

Genes involved in MHC class I APP play a role in different cancers [18] and may be considered candidate genes to estimate BC risk. Therefore, it is critical to assess the impact of functional polymorphisms on APP gene transcriptional activity and promoter polymorphisms on gene over- or under- expression. In the present study, we aimed to determine whether genes known to be associated with cancer immunosurveillance and immune evasion [19, 20] may be linked with BC risk and recurrence. Therefore, we investigated the association between MHC class I APP genes [single nucleotide polymorphisms (SNPs) and mRNA expression levels] and BC occurrence and recurrence. We selected nine SNPs in six genes (*ERAP1*, *ERAP2*, *TAP1*, *TAP2, TAPBP*, and *IFNG*) from the NCBI SNP database. The SNPs were analyzed in 124 BC patients and 503 healthy subjects from the European population from 1000 Genomes Project Phase 3.

The expression profile of the components of MHC class I APP in the course of BC and its correlation to recurrence is still missing. Differential expression of genes responsible for MHC class I APP in BC tumor tissues and non-tumor adjacent tissues may provide a molecular basis of the recurrence mechanism. Moreover, the gene expression level of the MHC class I APP components may serve as a prognostic indicator during the monitoring of BC patients after TURBT. The mRNA expression of *ERAP1*, *ERAP2*, *TAP1*, *TAP2*, and *TAPBP* was analyzed in 60 tumor tissues and corresponding non-tumor adjacent tissues, of which 30 tumors were with recurrence and thus collected twice. We quantified the effects of these SNPs on the mRNA expression of the corresponding genes. We performed TaqMan genotyping and quantitative real-time PCR (qRT-PCR), based on real-time PCR, the most reliable and widely used method. Additionally, we used ELISA to determine the association between plasma HLA and VEGF levels and recurrence. The obtained results point to a possible role of the MHC class I APP in BC risk and recurrence.

Patients and methods

Ethics. The research project was approved by the Ethics Committee of the Nofer Institute of Occupational Medicine (approval no. 01/2011 and 05/2019). It was implemented in accordance with the Declaration of Helsinki that defines procedures in human biomedical research. All experiments were performed following relevant guidelines and regulations. Subjects gave written informed consent to participate in the study.

Experimental design. The study consisted of 124 BC patients and 20 healthy controls (Figure 1). Patients with BC were recruited from the First Department of Urology, Medical University (Lodz, Poland) in 2011–2015. Healthy controls were recruited from the Nofer Institute of Occupational Medicine (Lodz, Poland), and blood was collected between 2013 and 2015. We used data from the European population of the 1000 Genomes Project (n=503). Patients with BC and controls were ethnically homogeneous Polish population or European population.

From all BC patients, we selected those with tissues collected (n=60). Patients were followed up for at least 2.5 years postoperatively with a median follow-up of 10 months (range 1–30 months) or until tumor recurrence if happened. The course of further observation is unknown. BC patients depending on recurrence before 10 months were stratified into two groups: "With recurrence" vs. "Without recurrence".

During the first collection hospital appointment and TURBT treatment, tissue and blood samples were collected as a "Primary tumor". The patients were monitored every 1–3 months afterward. During the second TURBT treatment, tissue and blood samples were collected from patients with "Recurrent tumor".

The characteristics of BC patients and controls such as gender, age, body mass index (BMI), and smoking status were obtained from questionnaires (with personal identifiers removed). For the smoking-stratified analyses, "non-smokers" were defined as subjects who were not smoking and had not smoked in the previous 5 years and "smokers" as those who smoked. The histological type was retrieved from the clinical files and classified according to the tumor staging (T) and grading (G). The patients were divided into three groups based on tumor stage: Ta, T1, T2, or tumor grade: well-differentiated (G1), moderately-differentiated (G2), poorly-differentiated (G3).

The data from the 1000 Genomes Project (Phase 3) were used as a control group for the gene association study. The

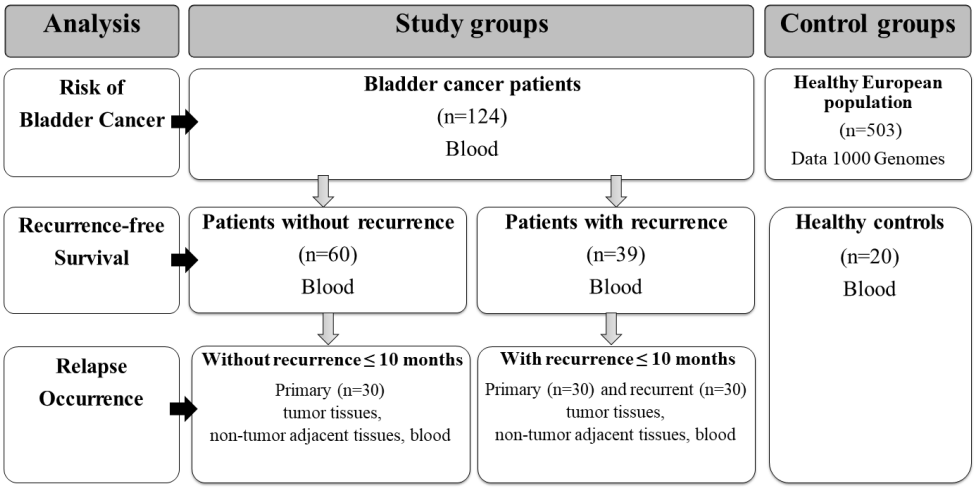

Figure 1. Study flowchart.

European population consisted of Utah Residents with Northern and Western European Ancestry, Toscani in Italy, Finnish in Finland, British in England and Scotland, and Iberian population in Spain.

Blood and tissue samples. Peripheral blood samples from 124 BC patients were collected into heparin-coated tubes and stored at –25 °C until genomic DNA isolation. From most of these patients (n=99), blood samples were collected during two visits: first collection „Primary tumor" from patients without (n=60) and with recurrence (n=39) and second collection „Recurrent tumor" from patients with recurrence (n=39) and healthy controls (n=20). Then, the plasma was obtained by centrifugation at 4,000 rpm for 10 min and stored at –25 °C until biochemical analyses.

A total of 180 tumor and non-tumor adjacent tissue samples were obtained during two visits. „Primary tumor" tissues were obtained during the first TURBT treatment from all 60 patients (30 patients without and 30 patients with recurrence of BC≤10 months). „Recurrent tumor" tissues were obtained during the second TURBT treatment from 30 patients with a recurrence of BC≤10 months. According to the manufacturer's instructions, tissue samples were collected and preserved in RNAlater Solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for RNA stabilization and storage. Samples were initially stored at 4 °C and immediately after delivery to the lab, archived at –25 °C until RNA isolation.

SNPs genotyping. Genomic DNA was isolated from the BC patients' peripheral blood using QIAamp DNA Mini Kits (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Genomic DNA was stored at –25 °C until analyses. Patients with BC were genotyped for the ten polymorphisms. Genotypes were analyzed by LightCycler 96 Real-Time PCR System (Roche Diagnostics, Switzerland) using TaqMan® SNP Genotyping Assays on demand (*ERAP1* rs1056893 ID:C_3282728_20, *ERAP2* rs251339 ID: C_2393507_10, *TAP1* rs1057141 ID: C_549926_20, *TAP1* rs1135216 ID: C_531909_20, *TAP2* rs241447 ID: C_175701925_10, *TAP2* rs241448 ID: C_2961793_10, *TAPBP* rs3106189 ID: C_2479333_10, *TAPBP* rs2071888 ID: C_11407883_1_, *IFNG* rs1861493 ID: C_2683476_10 Thermo Fisher Scientific). The LightCycler® 96 thermal cycler software performed the genotyping analysis. The data from the European population of the 1000 Genomes Project (Phase 3) were used as a control group for the genes tested. Candidate genetic polymorphisms were selected from the NCBI SNP database (https://www.ncbi.nlm.nih.gov) based on the high minor allele frequency higher than 5% and by a literature review [21–27], considering their association with BC and potential functionality [28].

mRNA expression. Total RNA was isolated from the BC tissue samples obtained during two visits using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Briefly, RNA was eluted in 30 µl RNase-free water and stored at –70 °C until use. The amount of the total extracted RNA was quantified by an Eppendorf BioPhotometer Spectrophotometer (Eppendorf, Hamburg, Germany). cDNA was synthesized from 100 ng of total RNA, using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacturer's protocol. The final volume of the reaction was 20 µl. Prepared cDNA was stored at –20 °C for further expression analysis.

mRNA expression was examined in BC tumor tissues and the matched non-tumor adjacent tissues. Quantitative real-time PCR (qPCR) was performed in duplicate for each sample using TaqMan Gene® Expression Assays (Thermo Fisher Scientific, South San Francisco, CA, USA) with FastStart Essential DNA Probes Master (Roche Diagnostics, Mannheim, Germany) on the LightCycler 96 Real-Time PCR System (Roche Diagnostics, Switzerland) following the

manufacturer's instructions. Candidate genes were selected based on known importance in the MHC class I APP.

mRNA expression profiles of six target genes were analyzed by several specific TaqMan Gene Expression Assays *ERAP1* (Hs00429970_m1), *ERAP2* (Hs01073631_m1), *TAP1* (Hs00388675_m1), *TAP2* (Hs00241060_m1), *TAPBP* (Hs00917451_g1), and *IFNG* (Hs00989291_m1, Hs99999041_m1). Ribosomal protein lateral stalk subunit P0 (*RPLP0*) and actin beta (*ACTB*) housekeeping genes were selected from commonly used reference genes (beta-2-microglobulin (Hs00187842_m1), phosphoglycerate kinase 1 (Hs00943178_g1), *RPLP0* (Hs00420895_gH), *ACTB* (Hs01060665_g1) for normalization of mRNA expression. A mean normalized expression value (MNE) was calculated from the Ct values obtained with the Q-Gene software.

Sandwich Enzyme-linked Immunosorbent Assay. Plasma HLA and VEGF levels were measured using specific Human HLA-A (Elabscience, E-EL-H1659) and Human VEGF (Invitrogen, Life Technologies, USA) ELISA kits, respectively. The Human HLA-A kit was used according to the manufacturer's instructions. The plasma samples were diluted twice with medium to bring the results into the linear portion of the standard curve to measure VEGF levels. The internal controls were included in each plate. The optical density was measured using a microplate reader Multiskan GO (Thermo Fisher Scientific, USA) at 450 nm. All samples were examined in duplicate, and the mean values were used for statistical analysis. The data were represented as means with the standard error of the mean (SEM).

Statistical analysis. The results are represented as mean ± standard deviation, if not stated otherwise. Normal distribution was verified by using the Shapiro-Wilk test. Chi-square tests, with appropriate corrections, were used to test associations between categorical variables. The Student's t-test was used for pairwise comparisons of continuous variables. Comparisons of continuous variables were analyzed using Mann-Whitney U tests due to non-normal distributions. For matched pair comparisons of ordinal variables, the Wilcoxon signed-rank test was used. One-way and two-way analysis of variance (ANOVA) and the Kruskal-Wallis were used for multiple-group comparisons. Further statistical analysis for post hoc comparisons was performed using the Dunn's test.

Data from patients with BC and the European population of the 1000 Genomes Project (Phase 3) were tested to determine whether the genotypes were in Hardy-Weinberg equilibrium (HWE). Observed genotype frequencies in patients and controls were compared with their expected frequencies at equilibrium based on the chi-square test with a statistical significance ($p<0.05$). The online Calculator of HWE https://wpcalc.com/en/equilibrium-hardy-weinberg/ as used.

The associations between selected polymorphisms and BC risk were estimated by odds ratios (ORs) and 95% confidence intervals (CIs) using logistic regression analysis. Major allele homozygotes served as the reference group for all polymorphisms, and heterozygotes and minor allele homozygotes were separately compared. Linkage disequilibrium (LD) as Pearson's squared correlation coefficient (r^2) was chosen to predict the LD between each pair of molecular markers. Genotypes were coded as 2, 1, and 0 in the function of the number of non-reference alleles.

A p-value of <0.05 was considered statistically significant. Statistical 12.0 software (StatSoft, Tulsa, USA) and GraphPad Prism 7 software (GraphPad Software, Inc., San Diego, CA, USA) were used for statistical analysis and data presentation.

Results

Characteristics of the study participants. The clinical and pathological characteristics of the study subjects are summarized in Table 1. The average age of BC patients was 62 years, and healthy controls was 67 years. More smokers were present among BC patients (41%) than among healthy controls (5%). BC patients and healthy controls differed in age and smoking status distribution ($p=0.0002$ and $p=0.005$, respectively). Males constituted 57% of BC patients and 75% of healthy subjects. There were no significant differences in BMI and gender between both groups.

Most of 124 BC patients analyzed for genetic polymorphism were classified as Ta (62%) fewer as T1 (23%) and T2 (15%). The distribution of tumor grade was as follows: well-differentiated (57%), moderately differentiated (28%), poorly differentiated (15%). Among 60 BC patients with analyzed mRNA expression and ELISA, Ta tumors were present in 66%, T1 in 27%, T2 in 7% cases, and G1 tumors in 60%, G2 in 30%, G3 in 10% cases. Moreover, small and single tumors were observed in 65% and 66% of BC patients, respectively.

IFNG genetic polymorphism is associated with an increased risk of BC. To assess whether genetic polymorphisms modulate BC risk, we analyzed the following polymorphisms: *ERAP1* rs1056893, *ERAP2* rs251339, *TAP1* rs1057141, rs1135216, *TAP2* rs241447, rs241448, *TAPBP* rs3106189, rs2071888, and *IFNG* rs1861493. We compared our results to the 503 data from the European population of the 1000 Genomes Project (Phase 3, n=503). Deviation from HWE was found for *TAP1* rs1057141 in the 1000 Genomes Project group and *TAP2* rs241447 and *TAPBP* rs2071888 in BC patients ($p<0.05$). The logistic regression model showed that *IFNG* rs1861493 was significantly associated with the BC risk (OR 1.94, 95%CI: 1.31–2.90). The BC patients carrying *GG* genotype showed an increased risk of BC occurrence ($p=0.001$, Table 2).

The number of tumors is associated with BC recurrence. In Supplementary Table S1, we compared the clinical and pathological characteristics of 99 BC patients and the occurrence of relapse during a 30-month observation. The median time interval of recurrence-free survival was 10 months (range 1–30 months). The number of tumors present in the patient was significantly associated with BC recur-

rence. BC patients with multiple tumors were more common among patients with recurrence ≤10 months (50%) than patients without recurrence ≤10 months (19%) (p=0.001). Kaplan-Meier curves showed that the number of tumors was significantly associated with recurrence-free survival (RFS) (p=0.010, Figure 2A).

Furthermore, there were no statistically significant differences between BC recurrence and other clinical and pathological characteristics such as age, BMI, smoking status, tumor stage, grade, or size. Among BC patients without recurrence, there were mainly cases with tumor stage Ta (72%), grade G1 (63%), and small tumor size ≤ 3 cm (70%).

TAPBP genetic polymorphisms are associated with the risk of BC recurrence. The logistic regression model showed that recurrence was associated with 2 SNPs in *TAPBP* (Supplementary Table S2). Both *TAPBP* rs3106189 and rs2071888 were independent risk factors for recurrence with OR 3.66 (p=0.014) and 2.76 (p=0.029), respectively. BC patients with the genotype *CC* in rs3106189 and *GG* in rs2071888 showed recurrence risk before 10 months.

Next, the rate of recurrence was assessed using the Kaplan-Meier estimation and compared by the log-rank test. We studied the relationship between genetic polymorphism of *ERAP1* rs1056893, *ERAP2* rs251339, *TAP1* rs1057141, rs1135216, *TAP2* rs241447, rs241448, *TAPBP* rs3106189, rs2071888, and *IFNG* rs1861493, and RFS using a Kaplan-Meier plotter. We found that the *TAPBP* rs2071888 *GG* genotype was associated with reduced RFS of BC patients (p=0.001, Figure 2B). Similarly, BC patients with *TAPBP* rs3106189 *CC* genotype had a shorter RFS than those with a *TT* or *CT* genotype (p<0.0001, Figure 2C).

Functional effects of SNPs in ERAP2 and TAP2. We examined a relationship between genetic polymorphism and expression of *ERAP1*, *ERAP2*, *TAP1*, *TAP2*, and *TAPBP* in the non-tumor adjacent tissue and tumor tissue samples by a two-way ANOVA test. We observed a correlation between *ERAP2* rs251339 and *ERAP2* expression (Figure 3A). Homozygote *TT* was associated with a high expression of the *ERAP2* (p=0.009). Moreover, we showed association between genetic polymorphism of *TAP2* rs241447 (p=0.007), rs241448 (p=0.012) and *TAP2* expression. We observed that homozygote *CC* in *TAP2* rs241447 (Figure 3B) and *GG TAP2* rs241448 (Figure 3C) were associated with high expression of *TAP2* regardless of tissue type.

Reduced level of TAPBP mRNA expression in tumor tissue. The expression of *ERAP1*, *ERAP2*, *TAP1*, *TAP2*, and *TAPBP* was determined in tumor and non-tumor adjacent tissues from 60 BC patients (Supplementary Table S3). Significantly lower *TAPBP* expression was observed in tumor tissue (2.279±0.331) compared with non-tumor adjacent

Table 1. Clinical and pathological characteristics of study groups. Values are mean ± standard deviation, or number of patients (%).

	BC patients[1] n=124 (100%)	BC patients[2] n=60 (100%)	Healthy controls[3] n=20 (100%)	p-value[4]
Age (years)	62.6±9.8	62.1±9.8	67.0±0.7	**<0.000**[a]
BMI (kg/m^2)	27.2±4.6	27.2±4.4	27.3±3.8	0.976 [a]
Gender				
Female	37 (30)	26 (43)	5 (25)	0.145[b]
Male	87 (70)	34 (57)	15 (75)	
Smoking status				
Non-smoker	82 (66)	37 (59)	19 (95)	**0.005**[b]
Smoker	42 (34)	23 (41)	1 (5)	
Tumor stage				
Ta	61 (62)	39 (66)		
T1	22 (23)	16 (27)	n/a	
T2	15 (15)	4 (7)		
Tumor grade				
G1	56 (57)	35 (60)		
G2	27 (28)	17 (30)	n/a	
G3	15 (15)	6 (10)		
Tumor size				
Small (≤3 cm)	70 (64)	39 (65)	n/a	
Large (>3 cm)	40 (36)	21 (35)		
Number of tumors				
Single	71 (65)	39 (66)	n/a	
Multiple	38 (35)	20 (34)		

Notes: [1]BC patients with the genetic polymorphism analysis performed; [2]BC patients with the gene expression and ELISA analyzes performed; [3]healthy controls with ELISA analysis performed; [4]p-values were determined by comparing the BC patients with mRNA and ELISA analyzes and healthy controls by [a]Student's t test or [b]Pearson χ^2-test; values in bold represent p<0.05; Abbreviations: BC-bladder cancer; BMI-body mass index; n/a: not applicable.

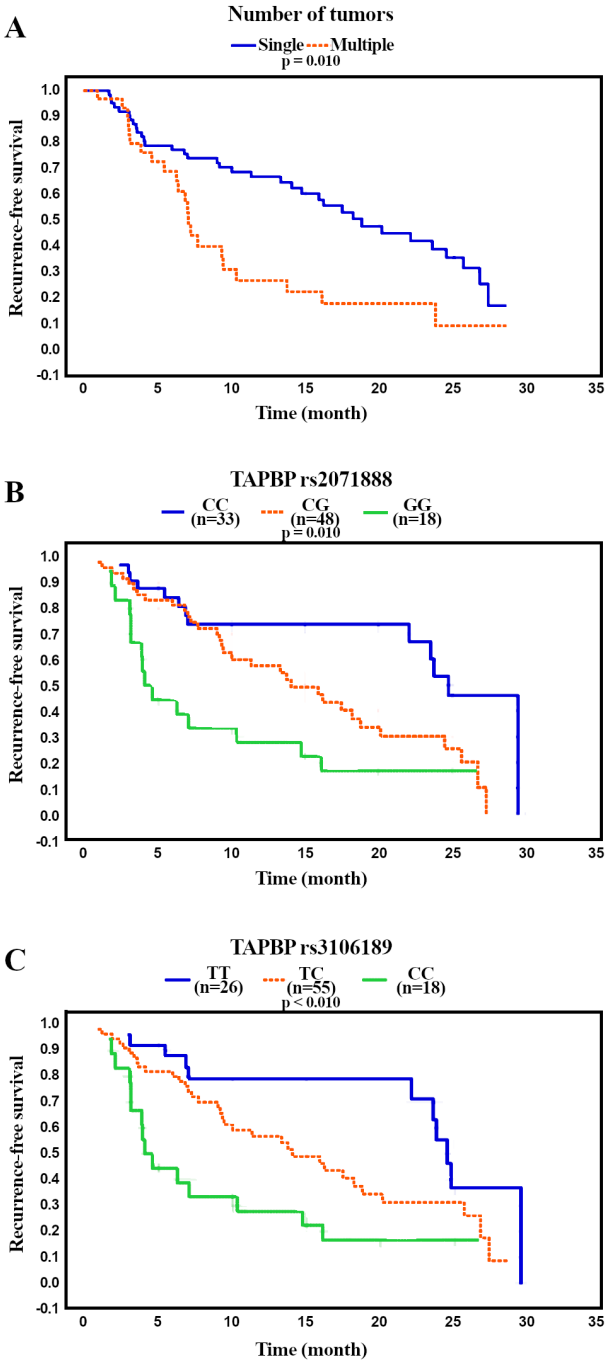

Figure 2. Kaplan-Meier curves present the association between A) the number of tumors; B) genetic polymorphisms *TAPBP* rs2071888; C) *TAPBP* rs3106189, and recurrence-free survival in patients with BC. The p-values were determined by log-rank (Mantel-Cox) test.

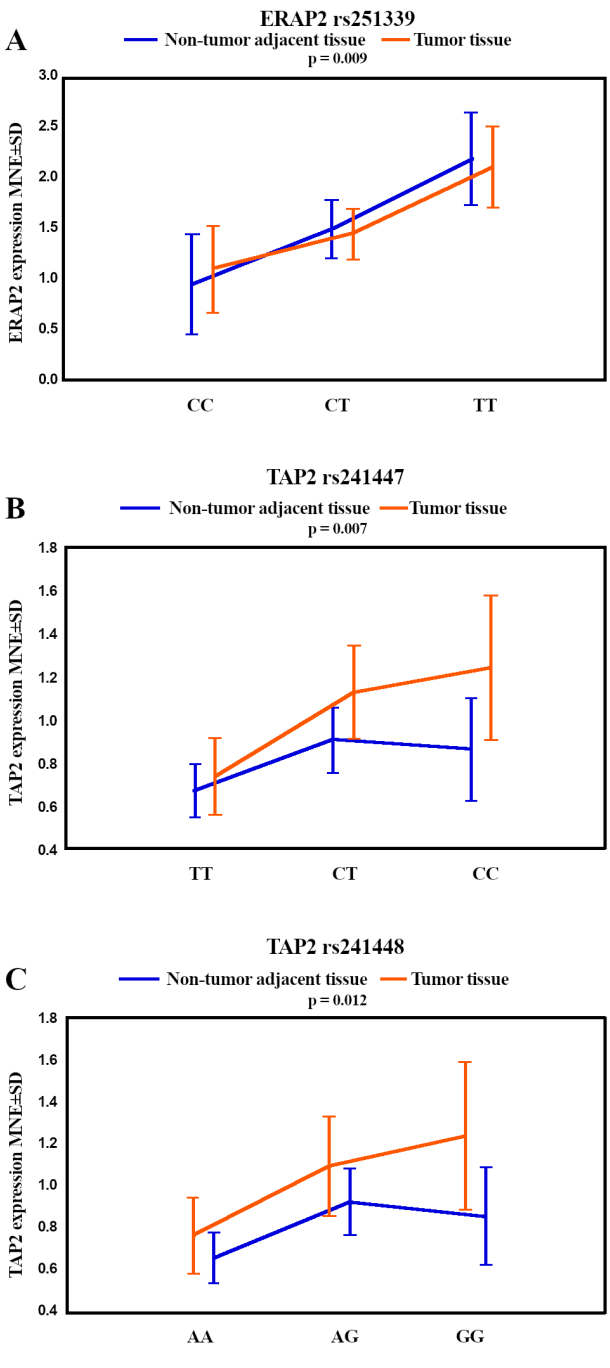

Figure 3. Association between genetic polymorphism and gene expression: A) *ERAP2* rs251339 and *ERAP2* expression; B) *TAP2* rs241447; C) *TAP2* rs241448 and *TAP2* expression. The comparison was made by two-way ANOVA.

tissue (2.364±0.192, p=0.042). Differences in the mRNA expression of the other genes did not differ between tissues.

mRNA expression profile of MHC class I APP components and clinicopathological features in the primary tumor. The mRNA expression levels of *ERAP1*, *ERAP2*, *TAP1*, *TAP2*, and *TAPBP* in tumor and non-tumor adjacent tissues were correlated with the clinical and pathological characteristics. There was no association between clinicopathological features and mRNA expression (p>0.05) in non-tumor adjacent tissues of BC patients with primary

Table 2. Selected single nucleotide polymorphisms and BC risk.

Gene SNP ID	Alleles Ref Alt	1000 Genomes Project* n = 503			BC patients n = 124			BC risk OR (95% CI), p-value
		Genotype: frequency (count)	MAF	HWE	Genotype: frequency (count)	MAF	HWE	
ERAP1 rs1056893	*TT*	0.268 (135)	0.48	0.57	0.226 (28)	0.50	1.16	Ref.
	CT	0.503 (253)			0.548 (68)			
	CC	0.229 (115)			0.226 (28)			
	CT+CC	0.732 (368)			0.774 (96)			1.25 (0.78-2.00), 0.362
ERAP2 rs251339	*CC*	0.298 (150)	0.46	0.18	0.242 (30)	0.48	2.15	Ref.
	CT	0.487 (245)			0.564 (70)			
	TT	0.215 (108)			0.194 (24)			
	CT+ TT	0.702 (353)			0.758 (94)			1.33 (0.85-2.08), 0.225
TAP1 rs1057141	*TT*	0.678 (341)	0.18	**0.03**	0.637 (79)	0.19	0.89	Ref.
	CT	0.292 (147)			0.339 (42)			
	CC	0.030 (15)			0.024 (3)			
	CT+CC	0.322 (162)			0.363 (45)			1.19 (0.79-1.80), 0.395
TAP1 rs1135216	*TT*	0.714 (359)	0.16	0.28	0.718 (89)	0.12	0.28	Ref.
	CT	0.258 (130)			0.266 (33)			
	CC	0.028 (14)			0.016 (2)			
	CT+CC	0.286 (144)			0.282 (35)			0.98 (0.64-1.49), > 0.999
TAP2 rs241447	*TT*	0.527 (265)	0.28	0.12	0.468 (58)	0.32	**0.02**	Ref.
	CT	0.393 (198)			0.427 (53)			
	CC	0.080 (40)			0.105 (13)			
	CT+CC	0.473 (238)			0.532 (66)			1.26 (0.84-1.87), 0.269
TAP2 rs241448	*AA*	0.527 (265)	0.28	0.12	0.516 (64)	0.30	1.61	Ref.
	AG	0.394 (198)			0.371 (46)			
	GG	0.080 (40)			0.113 (14)			
	AG+GG	0.473 (238)			0.484 (60)			1.04 (0.70-1.53), 0.841
TAPBP rs3106189	*TT*	0.264 (133)	0.50	2.43	0.274 (34)	0.46	0.36	Ref.
	CT	0.465 (234)			0.524 (65)			
	CC	0.270 (136)			0.202 (25)			
	CT+TT	0.736 (370)			0.726 (90)			0.95 (0.61-1.47), 0.825
TAPBP rs2071888	*CC*	0.286 (144)	0.48	2.94	0.323 (40)	0.43	**< 0.00**	Ref.
	CG	0.462 (232)			0.492 (61)			
	GG	0.252 (127)			0.185 (23)			
	CG+GG	0.714 (359)			0.677 (84)			0.84 (0.55-1.28), 0.441
IFNG rs1861493	*AA*	0.534 (269)	0.27	0.19	0.371 (46)	0.40	0.06	Ref.
	AG	0.388 (195)			0.468 (58)			
	GG	0.078 (39)			0.161 (20)			
	AG+GG	0.466 (234)			0.629 (78)			**1.94 (1.31-2.90), 0.001**

Notes: *analysis of the 503 data sets from European population of the 1000 Genomes Project (Phase 3); values in bold represent p<0.05
Abbreviations: MAF-minor allele frequency; HWE-Hardy-Weinberg equilibrium p-value; OR-odds ratio; 95% CI-95% confidence interval; Ref-reference allele; Alt-alternative allele

tumors (Supplementary Table S4). In tumor tissue of BC patients with primary tumor mRNA expression correlated with the number of tumors, smoking status, and BC recurrence (Table 3). Expression of *ERAP1* was higher in single compared with multiple tumors (p=0.024), while higher levels of *ERAP2* expression were identified in non-smoking patients than smokers (p=0.033). Higher levels of *TAP2* expression were found in patients with BC recurrence compared with patients without recurrence (p=0.009). We have not demonstrated a relationship between the expression of other genes and clinical or pathological features (p>0.05).

Changes in TAP2 mRNA expression between primary and recurrent tumors. The expression levels of *ERAP1*, *ERAP2*, *TAP1*, *TAP2*, and *TAPBP* were compared in non-tumor adjacent tissues and tumor tissues between primary and recurrent tumors. In non-tumor adjacent tissues, the *TAP2* expression level was significantly higher in recurrence (p=0.006). In tumor tissue, the *TAP2* expression level was also increased in recurrence but without statistical significance. There were no significant differences in the expression of *ERAP1*, *ERAP2*, *TAP1*, and *TAPBP* between primary and recurrent tumors (Supplementary Table S5).

Table 3. Gene expression in tumor tissues of BC patients with primary tumor.

	ERAP1[a] MNE ± SD	*ERAP2*[a] MNE ± SD	*TAP1*[a] MNE ± SD	*TAP2*[a] MNE ± SD	*TAPBP*[b] MNE ± SD
Gender					
Female	0.787±0.243	1.388±0.661	1.588±0.318	0.977±0.330	2.199±0.413
Male	0.765±0.292	1.518±0.649	1.464±0.438	0.818±0.446	2.289±0.341
Smoking status					
Non-smoker	0.791±0.270	**1.608±0.671**	1.579±0.397	0.910±0.383	2.248±0.402
Smoker	0.748±0.275	**1.233±0.559**	1.418±0.378	0.844±0.445	2.257±0.328
Tumor stage					
Ta	0.762±0.282	1.443±0.634	1.499±0.369	0.828±0.413	2.263±0.377
T1 and T2	0.784±0.232	1.494±0.740	1.544±0.478	1.041±0.374	2.208±0.375
Tumor grade					
G1	0.781±0.273	1.452±0.625	1.445±0.396	0.837±0.385	2.227±0.375
G2 and G3	0.724±0.251	1.395±0.664	1.591±0.375	0.963±0.449	2.251±0.357
Tumor size					
Small (≤3 cm)	0.768±0.242	1.473±0.587	1.509±0.378	0.886±0.374	2.327±0.216
Large (>3 cm)	0.786±0.318	1.447±0.764	1.526±0.429	0.878±0.459	2.128±0.520
Number of tumors					
Single	**0.830±0.236**	1.377±0.686	1.492±0.376	0.872±0.397	2.244±0.418
Multiple	**0.656±0.311**	1.597±0.543	1.553±0.446	0.896±0.447	2.261±0.265
Recurrence before 10 months					
Without recurrence	0.799±0.229	1.436±0.637	1.438±0.383	**0.757±0.371**	2.275±0.332
With recurrence	0.756±0.291	1.492±0.685	1.627±0.403	**1.052±0.402**	2.219±0.444

Notes: values are mean of Mean Normalized Expression (MNE) ± standard deviation (SD) of MNE; p-values were determined by [a]Student's t-test or [b]Mann-Whitney U test; values in bold represent p<0.05

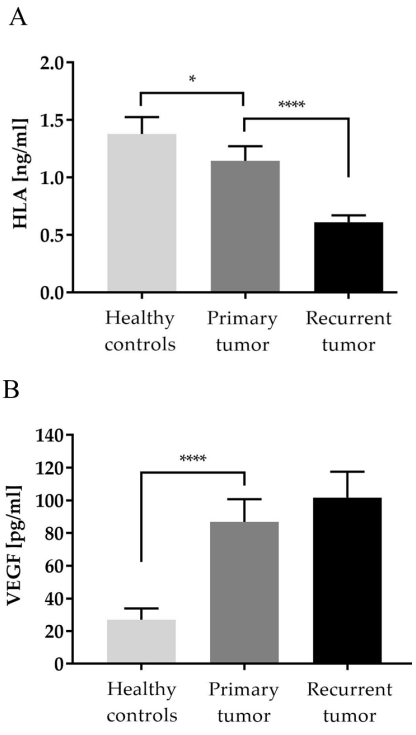

Figure 4. The difference in plasma A) HLA, B) VEGF protein levels in healthy controls and BC patients. Values are means with standard errors of the mean (SEM) determined by Kruskal-Wallis with Dunn's post hoc test (*p<0.05, ****p<0.0001).

Levels of HLA and VEGF in plasma. HLA and VEGF protein levels were determined in plasma from 20 healthy controls and 60 BC patients (Figure 4). The Kruskal-Wallis by ranks revealed statistically significant differences between groups regarding HLA and VEGF protein levels (p=0.006 and p<0.0001, respectively). The HLA protein levels were significantly lower in BC patients with primary tumors than healthy controls (p<0.05). In contrast, the VEGF protein levels were significantly higher in BC patients with primary tumors than healthy controls (p<0.0001). Importantly, HLA was lower (p<0.0001, Figure 4A), while VEGF levels were higher (p>0.05) in the recurrent tumor compared with the primary tumor (Figure 4B).

Lack of association between levels of HLA and VEGF and the clinicopathological features. The correlation analysis of HLA and VEGF protein levels and the clinicopathological features is summarized in Supplementary Table S6. Protein levels were determined in plasma from 60 BC patients with primary tumors. We have not demonstrated a significant association between the HLA, VEGF protein levels, and clinical or pathological features (p>0.05).

Discussion

MHC class I molecules are important for a robust anti-cancer immune response by presenting cancer antigens and activating T cells. Excellent reviews present a detailed description of T cell dysfunction in cancer and the need to

activate T cells in cancer immunotherapy (e.g., with checkpoint inhibitors) [29, 30]. There are low levels of tumor-infiltrating CD8+ T cells in BC. The lower tumor immunogenicity results in tolerance, allowing the tumor to escape immune recognition, recurrence, and progression [20] and could be caused by suboptimal antigen presentation by MHC class I molecules [31, 32]. Previous studies analyzed the mechanism of tumor escape from immune surveillance and demonstrated the downregulation of MHC class I molecules in tumor cells [33, 34]. It has been suggested that the SNPs in MHC class I APP could be used as clinical prognostic markers, which may aid decisions on further treatment. We conjectured those genes involved in these events could be regarded as candidate genes for BC risk. To our knowledge, this is the first research to analyze these SNPs in BC risk and recurrence. Previous studies showed the association between SNPs in *ERAP1* rs1056893, *ERAP2* rs251339, *TAP*1 rs1057141, rs1135216, *TAP*2 rs241447, rs241448, *TAPBP* rs3106189, rs2071888, *IFNG* rs1861493 and risk of various cancers [21–26]. We found an association between the genetic polymorphism of *IFNG* rs1861493 and the risk of BC. The BC patients carrying *GG* genotype showed an increased BC risk. This SNP was located on chromosome 12 68551196 intron variant c.366+497C>T. IFNG is a cytokine that stimulates *MHC class I, TAP1*, *TAP2, TAPBP, ERAP1,* and *ERAP2* [35]. It is conceivable that changes in the expression of this protein could increase the susceptibility to BC. However, the functional alteration caused by this SNP remains unknown. The present data indicate that others examined polymorphisms do not contribute to BC risk. Moreover, our results did not confirm the association between *TAP2* rs241447 and cancer risk in Caucasians shown by Liu et al. [26].

A previous study showed an association between the HLA I expression and RFS in BC. RFS was significantly longer for MHC class I-positive MIBC [36]. Moreover, BC patients with MHC class I-positive tumors had better RFS than those with MHC class I-negative tumors in the 5-year follow-up [33]. It can be assumed that the high recurrence risk is associated with a particularly adverse effect on the immune system of BC patients. The immune system can effectively eliminate MHC class I-positive tumors, and patients remain free from recurrence. A relationship between genetic polymorphisms of *TAPBP* rs3106189 and overall survival was identified in colorectal cancer [27]. Therefore, we suggest that it is crucial to examine the MHC class I APP major genes in BC recurrence. In our study, the SNPs in *TAPBP* were related to the BC recurrence probability. The rs3106189 at the 5' UTR of *TAPBP* is located on chromosome 6 33282002 intron variant c.607+180A>G. Moreover, the functional consequences of this SNP are associated with its strategic location within histone marks and among binding sites for transcription factors like interferon regulatory transcription factors 1, 2, 7, suggesting its role in transcriptional gene regulation. Importantly, we showed that subjects with the *CC* genotype were more susceptible to early (≤10 months) recurrence of BC than those with the combined *CG/GG* genotypes. In the *TAPBP* rs2071888 located on chromosome 6 33272855, missense variant c.779C>G, nucleotide substitution that results in an amino acid change at position 260 (threonine to arginine), we observed that *GG* genotype is a high-risk factor for an early (≤10 months) BC recurrence. Our results show that BC patients with the *TAPBP* rs3106189 *CC* and rs2071888 *GG* genotypes exhibited shorter RFS. Thus, these genotypes may be useful prognostic factors for BC recurrence. Moreover, we have found that these SNPs exist in potential LD relationships (r^2=0.775).

Genetic polymorphisms may determine mRNA expression levels [37, 38]. The introns may harbor functional SNPs that can influence the mRNA expression of the genes that host them. The intronic polymorphisms may modulate the genotype-phenotype relationship and confer susceptibility to disease [39]. This study analyzed the correlation between potentially functional SNP and mRNA expression of MHC class I APP components in BC tissues. The relationship between mRNA expression and genotype was evident in tumor and non-tumor adjacent tissue samples. We found that *ERAP2* gene rs251339 located on chromosome 5 96235038 intron variant c.1504-787T>C, the *TT* genotype was associated with high expression of *ERAP2*. In BC, tissue with the type of homozygotes of minor alleles had a higher level of *ERAP2* expression than carriers of heterozygotes minor alleles or homozygotes of major alleles. We also demonstrated that BC patients who were carriers of homozygotes of minor alleles of *TAP2* gene rs241447 or rs241448 had a higher level of *TAP2* mRNA expression compared with carriers of heterozygotes minor alleles or homozygotes of major alleles. In the *TAP2* gene rs241447 located on chromosome 6 32796751 with missense variant c.1993G>A, *CC* genotype remained a factor for high-level mRNA expression *TAP2*. In the *TAP2* gene rs241448 located on chromosome 6 32796685 with nonsense c.2059C>T, the *GG* genotype was a factor for high-level *TAP2* mRNA expression. These SNPs found to have potential LD relationships (r^2=0.850). However, despite the relationship between higher tissue *TAP2* mRNA expression and *CC* or *GG* genotypes, and the association of early recurrence with increased *TAP2* mRNA in primary tumor tissue (presented later in the discussion), these genotypes did not increase the risk of BC occurrence or recurrence. The functional significance of genetic polymorphism *ERAP2* (rs251339) and *TAP2* (rs241447 and rs241448) that influence gene expression in bladder tissue remains unknown. Our results were compared to allelic discrimination with log2-normalized expression data of those genes across different human tissues from the Genotype-Tissue Expression (GTEx). Expression quantitative trait loci (eQTLs) provided similar associations of genetic variants with gene expression for other tissues such as lung, thyroid, whole blood, and breast mammary tissue (https://gtexportal.org/home/snp/rs251339). Other mechanisms can also regulate gene expression, including posttranscriptional regulation by forming the miRNA-mRNA complexes or

transcriptional, posttranscriptional, and epigenetic control by lncRNAs [40].

Genetic markers are needed to complement the assessment of patients' clinical status. To define the potential role of the expression profile of genes essential for MHC class I APP, tumor tissue and matched non-tumor adjacent tissue from BC patients were obtained. The expression levels of *ERAP1, ERAP2, TAP1, TAP2*, *TAPBP* were assessed. In the present study, *TAPBP* expression in BC patients was lower in tumor tissue than in non-tumor adjacent tissues, indicating that the gene expression levels in tumor tissue may produce an insufficient amount of tapasin to ensure proper maturation of MHC class I molecules. TAPBP acts as a bridge between TAP and MHC class I molecules. Therefore, TAPBP protein levels and translocation of the peptides to the endoplasmic reticulum are critical factors in MHC class I molecules' assembly and surface presentation [41]. The function of *TAPBP*, a specific chaperone of MHC class I molecules, is to stabilize peptide-free forms and help MHC class I optimize peptide repertoire [42, 43]. The absence of TAPBP in mice impairs MHC class I expression and T cell development. Besides, inhibition of *TAPBP* gene transcription alters antigen presentation [44, 45].

The expression levels of *ERAP1*, *ERAP2*, *TAP1*, *TAP2,* and *TAPBP* in tumor tissues were dependent on some of the clinical and pathological characteristics. We observed statistically significant differences in mRNA expression in tumor tissues related to the number of tumors. BC patients with a single tumor had higher expression of *ERAP1* in agreement with the previous observation that expression of ERAP1 and ERAP2 was frequently altered in tumors [46]. Moreover, we also observed a link between the expression of *ERAP2* in tumor tissue and smoking status. The non-smoking BC patients had higher levels of *ERAP2* expression than smokers. The analysis showed that BC patients with early recurrence had increased *TAP2* expression in primary tumor tissue compared with BC patients without recurrence. It suggests that *TAP2* overexpression in tumor tissue may result from ineffective *TAP2* downregulation that recruits more immune infiltrates and modulates the inflammatory network in cancer development. Therefore, TAP1 and TAP2 overexpression may be an indicator of an aggressive breast tumor [47]. Importantly, the loss of TAP function results in diminished peptide supply and reduced cell surface levels of MHC class I molecules [48]. Additionally, downregulation of TAP is essential for cytotoxic T-lymphocyte recognition [49] and regulates repertoires of peptides presented MHC class I molecules [50].

The expression levels of genes were compared in primary and recurrent tumors. A statistically significant difference was observed in the *TAP2* mRNA expression in non-tumor adjacent tissues between the primary and recurrent tumors. We demonstrated increased *TAP2* mRNA expression in the recurrent tumor compared to the primary tumor. This relationship was not confirmed in tumor tissue. Therefore, changes in the expression of the *TAP2* gene may enable a more efficient antigen supply in non-tumor adjacent tissues. Additionally, we determined plasma levels of HLA and prognostic biomarker VEGF. The VEGF is one of the factors involved in the mediation of tumor angiogenesis and is considered a central player in this process. Several studies have demonstrated that VEGF activity is associated with tumor progression [51, 52]. VEGF has been suggested to plays a role in immunomodulation [53]. Moreover, anti-angiogenic therapy is a promising strategy against urothelial cancer initiation and progression [54]. Our study found no relationship between HLA or VEGF plasma levels and clinicopathological features. However, our results show higher VEGF levels and lower HLA levels in BC patients than healthy controls. Furthermore, among BC patients, low HLA and high VEGF levels were observed in recurrent tumors compared with the primary tumor. Our analysis is consistent with the previous findings related to VEGF and HLA levels. The high level of VEGF shows tumor development. In contrast, low HLA levels may represent an escape from the host immune pressure or may reflect the accumulation of abnormalities associated with tumor progression [50]. The evasion of the immune response is likely to occur within the BC because tumor cells do not usually express MHC class I molecules. Functionally inactive MHC class I molecules do not trigger effector functions of cytotoxic T lymphocytes. As a result, HLA and VEGF have come to represent critical diagnostic targets for BC.

Our research confirms previous studies that indicated the number of tumors, prior recurrence rate, and tumor grade as the most important prognostic factors for BC early recurrence. The number of tumors was one of the most critical factors for time to recurrence in BC patients [55, 56]. Similarly, we observed that the number of tumors present in the BC patients was associated with RFS. In addition, multiple tumors were associated with earlier recurrence.

Our findings indicate that SNPs and mRNA expression of MHC class I APP components may be associated with BC. As far as we are aware, this is the first study reporting functional effects of SNPs on mRNA expression in bladder tissue, and the association of MHC class I APP genes with BC risk of occurrence, recurrence and RFS. However, additional prospective studies with larger numbers of BC patients and longer follow-up periods are warranted to confirm the clinical importance of the MHC class I APP genetic factors in BC. New available genetics-based techniques allow exploring the functionality of genetic variants. Combined with the information about the environmental and behavioral factors modifying BC risk, these methods may allow the development of more comprehensive BC diagnostics and therapy.

Supplementary information is available in the online version of the paper.

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Genetic contributions of MHC class I antigen processing and presentation pathway to bladder cancer risk and recurrence

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Supplementary Information

Supplementary Table S1. Clinical and pathological characteristics of BC patients and the occurrence of relapse during 30 months observation

	BC patients without recurrence n=60 (100%)[1]	BC patients with recurrence n=39 (100%)[1]	p-value[2]
Age (years)	61.3±12.3	63.7±7.1	0.289[a]
BMI (kg/m^2)	26.5±4.6	27.7±4.4	0.195[a]
Gender			
Female	20 (33)	14 (38)	0.79[2]b
Male	40 (67)	25 (62)	
Smoking status			
Non-smoker	40 (67)	25 (62)	0.792[b]
Smoker	20 (33)	14 (38)	
Tumor stage			
Ta	33 (72)	21 (58)	0.203[b]
T1 and T2	13 (28)	15 (42)	
Tumor grade			
G1	29 (63)	20 (56)	0.492[b]
G2 and G3	17 (37)	16 (44)	
Tumor size			
Small (≤3 cm)	38 (70)	22 (58)	0.216[b]
Large (>3 cm)	16 (30)	16 (42)	
Number of tumors			
Single	43 (81)	19 (50)	**0.001**[b]
Multiple	10 (19)	19 (50)	

Notes: values are mean±standard deviation, or number of patients (%); [1]number of patients may vary due to missing data; [2]p-values were determined by [a]Student's t test or [b]Pearson χ^2-test; values in bold represent p<0.05; Abbreviations: BC-bladder cancer; BMI-body mass index; n/a-not applicable

Supplementary Table S2. Selected single nucleotide polymorphisms and risk of BC recurrence.

Gene SNP ID	Alleles Ref Alt	BC patients without recurrence n=60		BC patients with recurrence n=39		Risk of recurrence OR (95% CI), p-value
		Genotype: frequency (count)	HWE	Genotype: frequency (count)	HWE	
ERAP1 rs1056893	*TT*	0.267 (16)		0.205 (8)		Ref.
	CT	0.517 (31)	0.07	0.564 (22)	0.64	
	CC	0.217 (13)		0.231 (9)		1.40 (0.56–3.73), 0.485
	CT+ CC	0.733 (44)		0.795 (31)		
ERAP2 rs251339	*CC*	0.283 (17)		0.205 (8)		Ref.
	CT	0.500 (30)	<0.00	0.615 (24)	2.09	
	TT	0.217 (13)		0.179 (7)		1.53 (0.61–4.01), 0.381
	CT+ TT	0.717 (43)		0.795 (31)		
TAP1 rs1057141	*TT*	0.583 (35)		0.667 (26)		Ref.
	CT	0.383 (23)	0.59	0.308 (12)	**0.07**	
	CC	0.033 (2)		0.026 (1)		0.70 (0.30–1.56), 0.404
	CT+ CC	0.417 (25)		0.333 (13)		
TAP1 rs1135216	*TT*	0.717 (43)		0.692 (27)		Ref.
	CT	0.250 (15)	0.23	0.308 (12)	1.28	
	CC	0.033 (2)		0.000 (0)		1.12 (0.48–2.76), 0.794
	CT+ CC	0.283 (17)		0.308 (12)		
TAP2 rs241447	*TT*	0.533 (32)		0.462 (18)		Ref.
	CT	0.367 (22)	0.56	0.410 (16)	0.23	
	CC	0.100 (6)		0.128 (5)		1.33 (0.57–2.90), 0.485
	CT+ CC	0.467 (28)		0.538 (21)		
TAP2 rs241448	*AA*	0.567 (34)		0.564 (22)		Ref.
	AG	0.333 (20)	1.30	0.333 (13)	0.91	
	GG	0.100 (6)		0.103 (4)		1.01 (0.45–2.18), 0.979
	AG+ GG	0.433 (26)		0.436 (17)		
TAPBP rs3106189	*TT*	0.350 (21)		0.128 (5)		**Ref.**
	CT	0.567 (34)	2.90	0.538 (21)	0.60	
	CC	0.083 (5)		0.333 (13)		**3.66 (1.31–9.56), 0.014**
	CT+ CC	0.650 (39)		0.872 (34)		
TAPBP rs2071888	*CC*	0.417 (25)		0.205 (8)		Ref.
	CG	0.500 (30)	0.93	0.462 (18)	0.14	
	GG	0.083 (5)		0.333 (13)		**2.76 (1.10–6.95), 0.029**
	CG+ GG	0.583 (35)		0.795 (31)		
IFNG rs1861493	*AA*	0.317 (19)		0.359 (14)		Ref.
	AG	0.467 (28)	0.19	0.487 (19)	0.01	
	GG	0.217 (13)		0.154 (6)		0.82 (0.35–2.00), 0.662
	AG+ GG	0.683 (41)		0.641 (25)		

Note: values in bold represent p<0.05; Abbreviations: HWE-Hardy-Weinberg equilibrium p-Value; OR-odds ratio; 95% CI-95% confidence interval; Ref-reference allele; Alt-alternative allele

Supplementary Table S3. Differences in gene expression in tissues from BC patients with primary tumor.

	ERAP1 **MNE±SD**	***ERAP2*** **MNE±SD**	***TAP1*** **MNE±SD**	***TAP2*** **MNE±SD**	***TAPBP*** **MNE±SD**
Non-tumor adjacent tissue	0.706±0.227	1.501±0.708	1.540±0.306	0.830±0.287	2.364±0.192
Tumor tissue	0.744±0.268	1.441±0.599	1.543±0.375	0.900±0.408	2.279±0.331
p-value	0.3876[a]	0.3378[b]	0.6443[b]	0.2787[a]	**0.0424**[b]

Notes: values are mean of Mean Normalized Expression (MNE)±standard deviation (SD) of MNE; p-values were determined by [a]Student's t-test or [b]Mann-Whitney U; values in bold represent p<0.05

Supplementary Table S4. Gene expression in non-tumor adjacent tissues of BC patients with primary tumor.

	ERAP1 [a] MNE±SD	*ERAP2* [b] MNE±SD	*TAP1* [b] MNE±SD	*TAP2* [a] MNE±SD	*TAPBP* [b] MNE±SD
Gender					
Female	0.672±0.257	1.377±0.898	1.517±0.261	0.789±0.229	2.357±0.174
Male	0.703±0.220	1.713±0.555	1.500±0.349	0.735±0.331	2.336±0.244
Smoking status					
Non-smoker	0.712±0.232	1.673±0.755	1.567±0.323	0.770±0.311	2.374±0.182
Smoker	0.613±0.245	1.198±0.624	1.342±0.174	0.736±0.204	2.268±0.273
Tumor stage					
Ta	0.647±0.228	1.421±0.810	1.486±0.305	0.780±0.275	2.352±0.184
T1 and T2	0.750±0.238	1.808±0.468	1.513±0.295	0.717±0.329	2.314±0.273
Tumor grade					
G1	0.690±0.232	1.455±0.840	1.491±0.310	0.718±0.325	2.345±0.169
G2 and G3	0.661±0.241	1.679±0.513	1.500±0.288	0.831±0.209	2.332±0.274
Tumor size					
Small (≤3 cm)	0.677±0.187	1.469±0.554	1.443±0.215	0.779±0.284	2.351±0.170
Large (>3 cm)	0.709±0.316	1.728±1.040	1.634±0.414	0.727±0.297	2.338±0.283
Number of tumors					
Single	0.661±0.240	1.404±0.779	1.447±0.278	0.706±0.256	2.317±0.218
Multiple	0.733±0.232	1.835±0.618	1.643±0.350	0.853±0.318	2.409±0.200
Recurrence before 10 months					
Without recurrence	0.673±0.231	1.560±0.537	1.399±0.233	0.736±0.298	2.314±0.244
With recurrence	0.696±0.256	1.568±0.939	1.619±0.357	0.814±0.286	2.385±0.194

Notes: values are mean of Mean Normalized Expression (MNE)±standard deviation (SD) of MNE; p-values were determined by [a]Student's t-test or [b]Mann-Whitney U

Supplementary Table S5. Gene expression changes between clinical tissue collections in BC patients with recurrence.

	ERAP1 MNE±SD	*ERAP2* MNE±SD	*TAP1* MNE±SD	*TAP2* MNE±SD	*TAPBP* MNE±SD
Non-tumor adjacent tissue					
Primary tumor	0.688±0.236	1.555±0.746	1.508±0.306	0.761±0.285	2.346±0.211
Recurrent tumor	0.744±0.209	1.395±0.632	1.607±0.303	0.986±0.230	2.401±0.143
p-value	0.396[a]	0.330[b]	0.212[b]	**0.006**[a]	0.399[b]
Tumor tissue					
Primary tumor	0.774±0.270	1.463±0.651	1.515±0.394	0.883±0.407	2.252±0.371
Recurrent tumor	0.675±0.255	1.392±0.464	1.607±0.324	0.940±0.417	2.342±0.204
p-value	0.125[a]	0.625[a]	0.309[a]	0.581[a]	0.430[b]

Notes: values are mean of Mean Normalized Expression (MNE)±standard deviation (SD) of MNE; p-values were determined by [a]Student's t-test or [b]Mann-Whitney U test; values in bold represent $p<0.05$

Supplementary Table S6. HLA and VEGF protein concentration in plasma of BC patients with primary tumor.

	HLA [ng/ml] Mean±SEM	VEGF [pg/ml] Mean±SEM
Gender		
Female	1.38±0.24	97.79±25.37
Male	0.95±0.12	77.65±14.11
Smoking status		
Non-smoker	1.26±0.18	92.15±19.36
Smoker	0.98±0.16	75.98±14.39
Tumor stage		
Ta	1.22±0.17	67.54±9.81
T1 and T2	0.93±0.16	117.80±34.24
Tumor grade		
G1	1.18±0.16	76.77±13.89
G2 and G3	1.08±0.22	96.85±27.05
Tumor size		
Small (≤3 cm)	1.18±0.17	85.82±20.13
Large (>3 cm)	1.07±0.17	88.92±14.02
No of tumors		
Single	1.17±0.16	83.48±14.20
Multiple	1.12±0.23	94.45±29.58
Recurrence before 10 months		
Without recurrence	1.12±0.19	78.30±16.12
With Recurrence	1.21±0.19	98.59±22.57

Notes: values are mean of concentration in plasma±standard error of the mean (SEM); p-values were determined by Mann-Whitney U test; values in bold represent $p<0.05$