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Original Article

Genetic variability in 13q33 and 9q34 is linked to aggressiveness patterns and a higher risk of progression of non-muscle-invasive bladder cancer at the time of diagnosis

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Objective

To identify single nucleotide polymorphisms (SNPs) associated with patterns of aggressiveness of non-muscle-invasive bladder cancer (NMIBC).

Patients and Methods

From January 2011 to December 2018, 476 patients with NMIBC were prospectively included. The first step aimed to identify SNPs associated with aggressiveness patterns (e.g. \geq pT1 or high-grade/Grade 3 or presence of carcinoma *in situ*) by analysing the data of a genome-wide association study (GWAS) on 165 patients with BC. The second step aimed to validate the SNPs previously identified, by genotyping the germline DNA of 311 patients with NMIBC.

Results

Overall, the median (interquartile range) age was 66 (58–75) years and the rate of patients with aggressive NMIBC was comparable between both groups (46% vs 46%, $P = 1$). GWAS data analysis identified four SNPs associated with an aggressive NMIBC (rs12615669, rs4976845, rs2989734, and rs2802288). In the validation cohort, the genotype CC of rs12615669, as well as age >70 years at the time of diagnosis were associated with aggressive NMIBC ($P = 0.008$ and $P < 0.001$, respectively). Genotyping of the entire cohort showed an association between aggressive NMIBC and the T allele of rs12615669 ($P = 0.0007$), the A allele of rs4976845 ($P = 0.012$), and the A allele of rs2989734 ($P = 0.007$). A significant association was also found for the entire cohort between the risk of progression and the A allele of rs4976845 ($P = 0.04$).

Conclusion

This two-phase study identified three SNPs (rs12615669, rs4976845, and rs2989734) associated with aggressive NMIBC and one SNP (rs4976845) associated with a higher risk of progression.

Keywords

single nucleotide polymorphism, bladder, neoplasm, genetics, progression, #BladderCancer, #blcsm, #uroonc

Introduction

In 2018, 550 000 patients were diagnosed with bladder cancer (BC) worldwide, and the incidence of this disease increases by 1% each year [1]. Among them, 70% of patients with non-muscle-invasive BC (NMIBC) have a recurrence within 5 years of diagnosis [2]. Although patients with NMIBC have an overall survival rate of 93% at 5 years, BC is known to have the highest rate of recurrence of all malignancies [3,4]. Within this heterogeneous population of patients with

NMIBC, it is crucial to identify those who are more likely to recur or progress to an invasive stage with worse prognosis. Currently, the risk of progression and recurrence is assessed using risk stratification scores and nomograms, based on the patient's medical history, and pathology characteristics of the transurethral resection of the bladder (TURB) specimen [2,5]. The European Organisation for the Research and Treatment of Cancer (EORTC) score [2], Spanish Urological Oncology Group (CUETO) score [6] and the risk sub-stratification by the International Bladder Consultation Group (IBCG) [7] predict

the risk of recurrence and progression based on clinical and pathological factors, but none of these include genetic data. As elegantly shown in the setting of breast cancer, recurrence and progression risk may vary depending on the genetic predisposition of each patient with NMIBC [8,9].

Previous genome-wide association studies (GWAS) have identified 13 BC susceptibility variants in various chromosomal regions (Table 1) [10–17]. Rothman *et al.* [14], as part of a secondary analysis of a GWAS, measured the association between some of these single nucleotide polymorphisms (SNPs) and a higher risk of progression (e.g. T1–T4 or Grade 3–4). One SNP (rs8102137) was associated with increased risk of developing high-grade BC ($P = 0.016$) and several SNPs were found to lower the risk of progression (rs9642880, $P = 0.08$; rs401681, $P = 0.084$; rs798766, $P = 0.019$; rs1014971, $P = 0.038$). However, these studies were initially designed to measure the susceptibility of multiple variants to BC and they included patients with invasive and non-invasive diseases [18].

In the era of targeted disease prevention, precise identification of the patients with NMIBC who are more likely to recur or

progress remains challenging. As such, the combination of genetic and clinical characteristics may help to better stratify the individual risk of recurrence and progression of NMIBC.

The purpose of the present study was to identify SNPs associated with aggressiveness patterns, recurrence, and progression of NMIBC.

Patients and Methods

Study Design and Population

From January 2011 to December 2018, we recruited 476 Caucasian patients with a median (interquartile range [IQR]) age of 66 (58–75) years from a single institution, who had a pathologically confirmed diagnosis of urothelial NMIBC. Patients with histological variants of urothelial or non-urothelial carcinoma were excluded. All patients provided written informed consent to participate in this study. The sample collection was approved by the Assistance Publique-Hôpitaux de Paris Ethics Committee.

Within this population, two cohorts were formed. First, a learning cohort (GWAS cohort) included 165 patients with urothelial carcinomas (BC or tumour of the upper urinary tract) of any Stage and Grade, for whom we had GWAS genetic data [19]. Patients in the GWAS cohort were genotyped for >500 000 tag SNPs. Second, a validation cohort included 311 patients with NMIBC and ≥ 1 year of follow-up after the first resection.

For each patient in the two cohorts, the following data were collected: sex, age at diagnosis, smoking exposure, body mass index (BMI), association with upper-urinary tract urothelial carcinoma (UTUC) before or after the diagnosis of NMIBC, the risk score according to the EORTC [2] and the pathological characteristics (Stage, Grade according to the WHO classification 2016 [20], carcinoma *in situ* [CIS], multifocal character, and weight of resection specimens).

Follow-up data included the number and type of postoperative bladder instillations, recurrence after the first tumour, the time interval between initial diagnosis and recurrence, the total number of recurrences, progression, the interval between initial diagnosis and progression, and the type of progression (Ta or T1 towards T2, Ta towards T1, low- to high-grade, presence of CIS). For patients who progressed, data about whether or not chemotherapy and/or cystectomy was/were performed were collected.

Definition of Aggressiveness, Recurrence and Progression

The aggressiveness was defined based on the patients' clinical data. It included the Stage and Grade of the tumours at initial diagnosis. Patients' tumours were considered as 'not

Table 1 SNPs associated with the risk of developing bladder cancer.

SNPs	Loci	Closest gene	At risk allele	Reference
rs11892031	2q37.1	UGT1A cluster Intron	C	Selinski <i>et al.</i> [16]
rs10936599	3q26.2	TERC ACTRT3 MYNN LRRC34 Intron	C	Figueroa <i>et al.</i> [15]
rs710521	3q28	TP63 Intergenic	A	Kiemenev <i>et al.</i> [12]
rs798766	4p16.3	TACC3 – Intron FGFR3 Intergenic	T	Kiemenev <i>et al.</i> [11]
rs401681	5p15.33	CLPTM1L Intron	C	Rafnar <i>et al.</i> [13]
rs1495741	8p22	NAT2 Intergenic	G	Rothman <i>et al.</i> [14]
rs9642880	8q24.21	CASC11 Intron	T	Kiemenev <i>et al.</i> [12]
rs2294008	8q24.31	PSCA Intron	T	Rothman <i>et al.</i> [14]
rs907611	11p15.5	LSP1 Intergenic	A	Figueroa <i>et al.</i> [15]
rs7238033	18q12.3	SLC14A1 Intron	C/T	Garcia-Closas <i>et al.</i> [17]
rs8102137	19q12	CCNE1 Intergenic	C	Selinski <i>et al.</i> [16]
rs6104690	20p12.2	None	A	Figueroa <i>et al.</i> [15]
rs1014971	22q13.1	APOBEC3A Intergenic	C	Selinski <i>et al.</i> [16]

ACTN3, actinin alpha 3; APOBEC3A, apolipoprotein B mRNA editing enzyme catalytic subunit 3A; CASC11, cancer susceptibility 11; CCNE1, cyclin E1; CLPTM1L, cleft lip and palate transmembrane protein 1; FGFR3, fibroblast growth factor receptor 3; LRRC34, leucine-rich repeat-containing 34; LSP1, lymphocyte-specific protein 1; MYNN, myoneurin; NAT2, N-acetyltransferase 2; PSCA, prostate stem cell antigen; SLC14A1, solute carrier Family 14 Member 1 (Kidd Blood Group); TACC3, transforming acidic coiled-coil-containing protein 3; TERC, telomerase RNA component; TP63, tumour protein P63; UGT1A, UDP glucuronosyltransferase Family 1 Member A Complex Locus. [Correction added on 28 October 2020, after first online publication: Some of the reference numbering has been changed in this version.]

aggressive' if the tumour stage was pTa, and if tumour Grade was low, 1 or 2. Conversely, they were considered as 'aggressive' if the tumour Stage was \geq pT1, if tumour Grade was grade 3 (high grade), or if CIS was present.

Recurrence was defined as any new cancer detected after initial treatment and progression was defined as an increase to Stage \geq T2 disease in the bladder, an increase from Stage Ta to T1 disease, or an apparition of CIS.

Genotyping

Genomic DNA was extracted from blood using a standardised protocol. Genotype assays were done using genotyping facilities at the Centre de Recherche sur les Pathologies Prostatiques. The genotyping of the SNPs selected after the first phase of the study was performed by the 5' nuclease PCR method using commercially available TaqMan[®] assays as previously described by our group [21]. Allelic discrimination was done using the ABI[™] 7500 Sequence Detector, and results were analysed using the System Detection Sequence (SDS) version, 2.0.6 and the TaqMan Genotyper (Applied Biosystems, Waltham, MA, USA).

Statistical Analysis

Descriptive Statistics

Categorical variables were analysed using the chi-squared test. Continuous variables were represented by the median as the measure of central tendency and analysed using the Mann–Whitney test to compare the distribution of key variables between the GWAS and validation cohorts.

Analysis of GWAS Data

The association study between tag SNPs and the aggressiveness pattern in the GWAS cohort was carried out in three steps. First, logistic regression analysis between the genotypes of each of the 503 415 SNPs and the aggressiveness was carried out. For this first step, statistical significance was considered at $P < 0.001$. Secondly, a study using probabilistic learning in Bayesian networks according to the Markov chain model was executed in order to identify the SNPs that were most significantly associated with aggressiveness. A probabilistic profile, as well as the relative importance of associations between genotypes and aggressiveness were sought using the Bayesia Lab 9.0 software (Bayesia S.A.S., Changé, France). The SNPs presenting the best association according to the Kullback–Leibler distance in the probabilistic model were selected. Thirdly, a new logistic regression analysis was completed to select the most significant SNPs for the validation phase, with consideration for allelic and genotypic frequencies [22]. The genotypes, the groupings of genotypes, and the alleles of each of the SNPs were analysed.

Bonferroni correction for multiple comparisons was applied and only $P < 0.00009$ was considered significant.

Analysis of Data of the Validation Cohort

Odds ratios (ORs), as a measure of relative risk, and 95% CIs were estimated using logistic regression models. All statistical analyses were carried out using XLSTAT 2020.3 (Addinsoft, New York, NY, USA) and StatView[™], version 5.0 software (SAS Institute, Cary, NC, USA).

Results

Study Population

Overall, 476 patients with a median (IQR) age of 66 (58–75) years and a male to female ratio of 4.2:1 were included between 2011 and 2018. Of these, 165 patients were included in the GWAS group and 311 patients were included in the validation group. The rate of pTa tumours was 63% ($n = 104$) in the GWAS group vs 66% ($n = 205$) in the validation group, with no significant difference ($P = 0.5$). The two groups were comparable in terms of tumour grade and tumour stage at diagnosis, recurrence and progression rates, and rate of post-TURBT intravesical instillations (Table 2). The median (IQR) number of recurrences was higher in the GWAS group than in the validation group, at 8 (6–8) vs 6 (6–8) ($P = 0.03$). The median (IQR) follow-up was significantly longer in the GWAS group, at 123 (68–154) vs 51.5 (31–103) months ($P < 0.001$). The proportion of patients with aggressive BC was comparable in both groups (46% vs 46%, $P = 1$).

GWAS Data Analysis

A first logistic regression analysis of the GWAS data obtained for 165 patients with urothelial cancer identified 544 SNPs that were most significantly associated ($P < 0.001$) with aggressiveness. From these 544 SNPs, a study using probabilistic learning in Bayesian networks according to the Markov chain model then identified 16 SNPs with the strongest statistical association with aggressiveness.

For these 16 SNPs, a new logistic regression analysis was performed taking into account alleles, genotypes, and their combinations (Table 3). For five of the variants (rs1954492, rs2393892, rs179618, rs2919747, and rs17767579), the results did not reach the threshold of $P < 0.00008$ considered after Bonferroni correction. For the other 11 variants, a significant association was found. Of these 11 variants, seven were excluded from the validation analysis because their allelic or genotypic frequency was below the fixed threshold of 16% (Table 3).

Finally, four SNPs were selected for the validation step: rs12615669, rs4976845, rs2989734, and rs2802288. For these

four markers, the most significant associations were as follows. The C allele of rs12615669 (OR 2.63, 95% CI 1.69–4.2; $P = 0.00002$), the combination of AA + GG genotype of rs4976845 (OR 4.44, 95% CI 2.17–9.07; $P = 0.00002$), the allele A of rs2989734 (OR 2.68, 95% CI 1.68–4.26; $P = 0.00003$) and the A allele of rs2802288 (OR 2.56, 95% CI 1.62–4.03; $P = 0.00004$) were associated with a greater risk of aggressive BC.

Validation Cohort Analysis

Genotyping of the four selected SNPs was then performed in the validation cohort of 311 patients with NMIBC. The frequencies of genotypes and alleles of the different polymorphisms were compared between the following groups of patients: aggressive or not aggressive NMIBC, with or without recurrence and, finally, with or without progression. All results are displayed in Table 4. A significant association was observed between the CC genotype of rs12615669 and aggressiveness (OR 2.02, 95% CI 1.19–3.4; $P = 0.008$). The

univariate analysis also showed a statistical association between an age >70 years at the time of diagnosis and aggressiveness (OR 2.74, 95% CI 1.73–4.34; $P < 0.001$). The Bayesian analysis shown in Fig. 1 confirmed this association between age and aggressiveness (Bayes factor = 1.32).

Global Cohort Analysis

A complementary association study conducted on data from the overall cohort identified three SNPs which were statistically associated with aggressiveness of NMIBC (Table 5). The C allele of rs12615669 (OR 1.57, 95% CI 1.21–2.04; $P = 0.0007$), the G allele of rs2989734 (OR 1.43, 95% CI 1.10–1.87; $P = 0.007$) and the A allele of rs4976845 (OR 1.39, 95% CI 1.07–1.80; $P = 0.012$) were associated with an increased risk of developing an aggressive tumour.

Moreover, the A allele of rs4976845 was significantly associated with an increased risk of progression (OR 1.52, 95% CI 1.02–2.28; $P = 0.04$).

Table 2 Demographics and characteristics of the study population.

	GWAS cohort (<i>n</i> = 165)	Validation cohort (<i>n</i> = 311)	Global cohort (<i>n</i> = 476)	<i>P</i>
Gender, <i>n</i> (%)				
Male	138 (84)	246 (79)	384 (81)	0.28
Female	27 (16)	65 (21)	92 (19)	–
Age at diagnosis, years, mean (SD)	63.9 (12.5)	66.9 (11.8)	65.8 (12.1)	0.011
Smoking status, <i>n</i> (%)				
Never	38 (24)	67 (23)	106 (22)	1
Active/former	123 (76)	219 (77)	342 (72)	–
Tumour stage after TURBT, <i>n</i> (%)				
Ta	104 (63)	205 (66)	309 (65)	0.5
≥T1	61 (37)	106 (34)	167 (34)	–
Multifocal tumours, <i>n</i> (%)				
Yes	52 (50)	103 (36)	155 (40)	0.02
Tumour Grade after TURBT, <i>n</i> (%)				
G1	36 (22)	81 (27)	118 (25)	0.23
G2	68 (42)	100 (33)	173 (36)	–
G3	57 (35)	119 (39)	176 (37)	–
CIS, <i>n</i> (%)	10 (8.6)	39 (13)	49 (10)	0.28
Aggressive tumour, <i>n</i> (%)*				
Yes	76 (46)	143 (46)	219 (46)	1
UTUC, <i>n</i> (%)				
No	78 (68)	242 (85)	320 (80)	<0.001
Before-NMIBC	15 (13)	24 (8.4)	39 (9.8)	–
After-NMIBC	22 (19)	19 (6.7)	41 (10)	–
Intravesical instillations, <i>n</i> (%)				
Yes	65 (64)	168 (55)	233 (50)	0.17
Number of instillations, median (IQR)	8 (6–8)	6 (6–8)	6 (6–8)	0.03
Type of instillation, <i>n</i> (%)				
BCG	34 (51)	113 (66)	148 (61)	0.057
Mitomycin C	33 (49)	55 (33)	91 (38)	–
Recurrence, <i>n</i> (%)	80 (67)	196 (63)	276 (58)	0.6
Number of recurrences, median (IQR)	2 (1–4)	2 (1–3)	2 (1–4)	0.009
Progression, <i>n</i> (%)	18 (19)	38 (13)	56 (12)	0.19
Cystectomy, <i>n</i> (%)	19 (17)	19 (6.1)	38 (8.9)	<0.01
Death, <i>n</i> (%)	6 (7.2)	26 (8.3)	32 (8.1)	0.93
Follow-up, months, median (IQR)	123 (68–154)	51.5 (31–103)	58 (31–109)	<0.001

*Aggressive tumour: ≥pT1 or Grade 3 or presence of CIS. Bold values statistically significant at $P < 0.05$.

Table 3 Logistic regression analysis of the 16 SNPs issued from the Bayesian network analysis.

SNPs	Loci	Gene (localisation)	Genotypes or alleles	Aggressiveness (-) N (%)	Aggressiveness (+) N (%)	OR (95% CI)	P
rs12615669	2p22.1	NA	T	103 (57.9)	52 (34.2)	Reference	0.00002
			C	75 (42.1)	100 (65.8)	2.63 (1.69–4.2)	
rs4976845	13q33.3	<i>MYO16</i> (intron)	AA	15 (16.9)	36 (47.4)	Reference	0.00002
			AG + GG	74 (83.1)	40 (56.6)	4.44 (2.17–9.07)	
rs2989734	9q34.3	<i>FCN1</i> (3' region, untranslated)	G	91 (51.1)	112 (73.7)	Reference	0.00003
			A	87 (48.9)	40 (26.3)	2.68 (1.68–4.26)	
rs158487	5q11.2	NA	C	148 (84.1)	97 (63.8)	Reference	0.00003
			T	28 (15.9)	55 (36.2)	3.00 (1.79–5.05)	
rs10737346	1q25.3	NA	GG	87 (97.8)	58 (76.3)	Reference	0.00003
			AA + AG	2 (2.2)	18 (23.7)	13.50 (3.02–60.39)	
rs202859	21q21.1	NA	A	138 (77.5)	143 (94.1)	Reference	0.00003
			G	40 (22.5)	9 (5.9)	0.22 (0.10–0.46)	
rs2802288	6q21	<i>FOXO3</i> (intron)	G	126 (70.8)	75 (48.7)	2.56 (1.62–4.03)	0.00004
			A	52 (29.2)	79 (51.3)		
rs1439570	5q23.2	<i>LINC02240</i> (intron)	T	116 (65.2)	129 (84.9)	0.33 (0.159–0.57)	0.00005
			C	62 (34.8)	23 (15.1)		
rs11874036	18q12.1	<i>GAREM1</i> (intron)	TC	160 (89.9)	152 (100)	Reference	0.00006
				18 (10.1)	0 (0)	0.03 (0.00–0.48)	
rs373612	16q23.1	NA	T	122 (68.5)	132 (86.8)	Reference	0.00008
			C	56 (31.4)	20 (13.2)	0.33 (0.19–0.58)	
rs1526709	3p13	NA	AA + AC CC	81 (94.2)	54 (71.1)	Reference	0.00008
				5 (5.8)	22 (28.9)	0.15 (0.05–0.43)	
rs1954492	14q23.2	<i>LINC00643</i> (intron)	GG	86 (97.7)	60 (80)	Reference	0.00022
			GT	2 (2.3)	15 (20)	10.75 (2.37–48.75)	
rs2393892	10q21.2	<i>ZNF365</i> (intron)	A	120 (67.4)	129 (84.9)	Reference	0.00024
			G	58 (32.6)	23 (15.1)	0.37 (0.21–0.64)	
rs1796180	12q21.31	<i>TMTC2</i> (intron)	AA	70 (78.7)	74 (97.4)	Reference	0.00032
			AG	19 (21.3)	2 (2.6)	0.10 (0.02–0.44)	
rs2919747	2q21	NA	AA	89 (100)	66 (66.3)	Reference	0.00041
			AG + GG	0 (0)	10 (13.2)	28.26 (1.63–490.92)	
rs17767579	14q23.3	<i>SPTB</i> (intron)	C	117 (65.7)	123 (82)	Reference	0.00092
			A	61 (34.3)	27 (18)	0.42 (0.25–0.71)	

FOXO3, Forkhead Box O3; *GAREM1*, *GRB2* associated regulator of *MAPK1* subtype 1; *LINC0(2240)(0643)*; long intergenic non-protein coding RNA (2240) (643); NA, not available; *SPTB*, spectrin beta, erythrocytic; *TMTC2*, transmembrane O-mannosyltransferase targeting cadherins 2; *ZNF365*, zinc finger protein 365. SNPs in bold: selected SNPs; Significant P values in bold: p value < significance threshold of 0.00008 after Bonferroni correction; Frequencies in bold: allele or genotype frequencies <16%.

Discussion

Using genotypic data from 503 415 tag SNPs from a GWAS involving 165 patients with urothelial carcinoma, we were able to identify 16 SNPs associated with the aggressiveness of NMIBC. Among these 16 SNPs only four (rs12615669, rs4976845, rs2989734, rs2802288) had an allelic or genotypic frequency high enough to be selected for the validation analysis performed on 311 patients with NMIBC. None of these four SNPs had been associated with the risk of developing a bladder tumour in previous GWAS studies [11,12,14–16,19].

The main result of our present study is the statistical association between rs12615669, rs4976845, rs2989734, and NMIBC aggressiveness. Among these three variants, the A allele of rs12615669 had the most significant association, with an increased risk of developing aggressive BC (OR 1.57, 95% CI 0.49–0.83; $P = 0.0007$). The variant rs12615669 had the fifth higher association with aggressiveness after the GWAS data analysis of 503 415 tag SNPs and was one of the two most significant SNPs after analysis in the Bayesian network

and logistic regression (OR 2.63, 95% CI 1.69–4.2; $P = 0.00002$). This result was confirmed by our validation study on an independent cohort of patients with NMIBC (OR 2.02, 95% CI 1.19–3.4; $P = 0.009$). No previous study has ever mentioned this SNP, which is not located in a known gene.

The variant rs2989734 is located in the 3' untranslated region of the ficolin 1 (*FCN1*) gene. This gene codes for a protein (Ficolin1), which has an elastin-binding activity and which could also be involved in immune modulation in addition to its pathogen recognition function [23]. The analysis of proteomic data obtained from the cohort of 877 TCGA (The Cancer Genome Atlas) patients showed that high expression of the *FCN1* protein was associated with decreased overall survival in kidney cancer [24], but this result has not yet been validated by a dedicated study. No other study has reported a link between this gene and BC. Expression quantitative trait loci (eQTL) analysis seeking to identify correlations of gene expression as a function of SNP genotypes has shown a correlation between the genotypes of rs2989734 and the level of expression of the *FCN1* gene [25].

Table 4 Association between genotypes for the four selected SNPs and aggressiveness, recurrences and progression for the validation cohort.

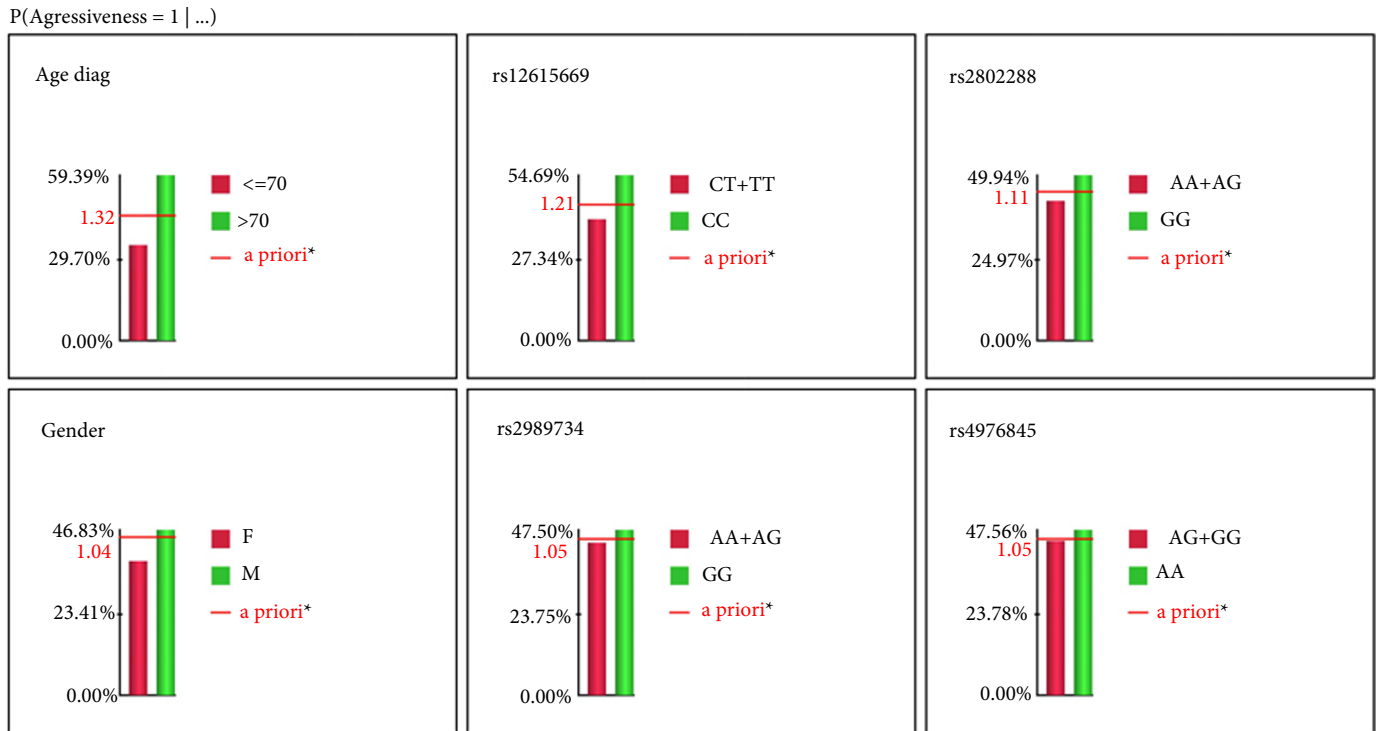
SNPs	Gene	Aggressiveness (-) N (%)	Aggressiveness (+) N (%)	OR	95% CI	P
Association between aggressiveness and genotypes						
rs12615669						
CT	NA	93 (57)	57 (42)	Reference		
CC		42 (26)	52 (38)	2.02	1.19–3.4	0.009
TT		28 (17)	27 (20)	1.57	0.84–2.93	0.15
rs4976845						
GG	MYO16	44 (27)	34 (24)	Reference		
AG		87 (52)	72 (52)	1.1	0.62–1.84	0.81
AA		35 (21)	33 (24)	1.22	0.64–2.35	0.55
rs2989734						
AG	FCN1	87 (52)	67 (48)	Reference		
GG		57 (34)	51 (37)	1.16	0.71–1.90	0.55
AA		24 (14)	21 (15)	1.13	0.58–2.21	0.70
rs2802288						
AG	FOXO3	83 (50)	63 (45)	Reference		
GG		55 (33)	56 (40)	1.34	0.82–2.20	0.25
AA		29 (17)	21 (15)	0.95	0.5–1.8	0.89
Association between recurrences and genotypes						
rs12615669						
CC	NA	36 (32)	58 (31)	Reference		
CT		57 (52)	93 (50)	1.01	0.60–1.72	0.96
TT		18 (16)	37 (19)	1.28	0.63–2.57	0.50
rs4976845						
GG	MYO16	30 (27)	48 (25)	Reference		
AG		57 (51)	102 (53)	1.12	0.64–1.96	0.70
AA		25 (22)	43 (22)	1.08	0.55–2.10	0.83
rs2989734						
GG	FCN1	37 (32)	71 (37)	Reference		
AG		60 (52)	94 (49)	1.22	0.73–2.05	0.44
AA		18 (16)	27 (14)	1.27	0.6–2.61	0.50
rs2802288						
GG	FOXO3	43 (38)	68 (35)	Reference		
AG		54 (48)	92 (47)	1.08	0.65–1.79	0.77
AA		16 (14)	34 (18)	1.34	0.66–2.72	0.41
Association between progression and genotypes						
rs12615669						
CC	NA	80 (31)	15 (39)	Reference		
CT		129 (50)	20 (53)	0.83	0.40–1.71	0.60
TT		50 (19)	3 (8)	0.32	0.09–1.16	0.07
rs4976845						
GG	MYO16	71 (27)	8 (21)	Reference		
AG		136 (52)	19 (49)	1.24	0.52–2.97	0.63
AA		57 (21)	12 (30)	1.87	0.72–4.88	0.20
rs2989734						
GG	FCN1	90 (34)	16 (42)	Reference		
AG		140 (52)	14 (37)	0.56	0.26–1.21	0.14
AA		37 (14)	8 (21)	1.22	0.48–3.09	0.68
rs2802288						
GG	FOXO3	97 (36)	13 (34)	Reference		
AG		131 (49)	15 (39)	0.85	0.39–1.88	0.70
AA		39 (14)	10 (26)	1.91	0.78–4.73	0.16

Significant *P* value < .05 in bold.

The GG genotype of the variant rs2989734 could result in a higher expression of this gene, as observed in adipose tissue, but that study did not include specific expression in bladder tissue. Therefore, the result of that eQTL analysis suggested that the role of this SNP on the aggressiveness or progression of BC could be mediated by its effect on the expression of FCN1.

The second main result of our present study is the association between the A allele of rs4976845 and an

increased risk of progression of NMIBC (OR 1.52, 95% CI 1.02–2.28; *P* = 0.04). The SNP rs4976845 is located in intron 33 of the myosin XVI (*MYO16*) gene. A recent study on rat cell lines suggested that this gene could have a regulatory role in the cell cycle [26]. The alteration of its expression could play a role in the carcinogenesis process, but no direct causal link has currently been established between this gene and the development of bladder tumours or their aggressiveness.

Fig. 1 Risks factor of aggressive NMIBC at diagnosis in the validation cohort. *Bayes factor is to be interpreted as a likelihood ratio of the marginal likelihood of two competing hypotheses, usually a null and an alternative.**Table 5** Association between alleles for the four selected SNPs and aggressiveness or progression for the global cohort.

SNPs	Gene	Aggressiveness (-) N (%)	Aggressiveness (+) N (%)	OR	95% CI	P
Association between aggressiveness and alleles						
rs12615669						
T	NA	127 (50)	82 (39)	Reference		
C		127 (50)	129 (61)	1.57	1.21–2.04	0.0007
rs4976845						
G	MYO16	136 (54)	96 (45)	Reference		
A		118 (46)	118 (55)	1.39	1.07–1.80	0.012
rs2989734						
A	FCN1	111 (43)	75 (35)	Reference		
G		147 (57)	139 (65)	1.43	1.10–1.87	0.007
rs2802288						
G	FOXO3	159 (62)	124 (58)	Reference		
A		98 (38)	90 (42)	1.21	0.93–1.57	0.16
Association between progression and alleles						
rs12615669						
C	NA	180 (54)	34 (61)	Reference		
T		153 (46)	21 (39)	0.76	0.50–1.14	0.18
rs4976845						
G	MYO16	172 (51)	23 (41)	Reference		
A		166 (49)	33 (59)	1.52	1.02–2.28	0.04
rs2989734						
G	FCN1	204 (60)	37 (68)	Reference		
A		136 (40)	18 (32)	1.45	0.94–2.22	0.09
rs2802288						
G	FOXO3	207 (61)	30 (55)	Reference		
A		133 (39)	25 (45)	1.26	0.84–1.89	0.27

Significant P value < .05 in bold.

Another interesting finding of the present study was the association between age and the aggressiveness of bladder cancer. In the 2006 EORTC study, Sylvester *et al.* [2] identified clinical and pathological factors associated with the risk of progression and/or recurrence. In the EORTC study, age was associated with the risk of progression (hazard ratio [HR] 1.36, $P = 0.012$), but not with the risk of recurrence (HR 1.10, $P = 0.09$). Age is known to be the main risk factor for developing a bladder tumour [27]. Several studies have also shown that age is a risk factor for developing higher stage and grade tumours [28,29]. In the present study, age at diagnosis was an independent factor associated with the severity of bladder tumours. This result may be explained by the fact that a later diagnosed disease increases the risk of an advanced natural history of the disease. Besides, exposure to toxicants (tobacco or occupational exposure) is a known risk factor for bladder tumours. The later the disease is diagnosed, the longer the exposure to these toxins and, therefore, the greater the risk of discovering a more serious disease. However, this association between age and aggressiveness might also reflect a treatment bias (i.e. not doing a cystectomy in older patients with a similar high-risk NMIBC presentation as younger patients).

During the secondary analysis of their GWAS data, Rothman *et al.* [14] observed that the C allele of rs8102137 was significantly associated with the risk of developing a high-grade tumour ($P = 0.016$) and with a non-significant higher risk of progression ($P = 0.08$). In the present study, analysis of the data from the GWAS of 165 patients with urothelial carcinoma did not find this association ($P = 0.23$ after the first logistic regression), nor those of the other markers that they had identified with a lower risk of progression, despite a very close classification to define aggressiveness. This discrepancy could be explained either because, unlike Rothman *et al.* [14], we considered CIS to be a criterion of aggressive NMIBC in our classification, or because our first logistic regression focussed on genotypes and not on alleles. Additionally, our present study on a population of 165 patients may not have had enough power to achieve statistical significance for a low OR of ~ 1.23 , as observed in their study.

These results should be interpreted in light of the study limitations. First, the limited size of the GWAS cohort did not allow the study of direct associations between the 503 415 SNPs and recurrence or progression. Thus, we used aggressiveness criteria that were very similar to those used by Rothman *et al.* [14] to define the risk of progression of BC. The few progression events and/or the small size of the validation cohort did not allow for the detection of significant results for progression, which suggests that if such associations exist, the corresponding ORs are lower than those obtained for aggressiveness and therefore do not allow their identifications on a population of this size. Our present study highlights that the search for SNPs linked specifically to recurrence and/or

progression of NMIBC requires targeted GWAS with larger numbers of patients with or without recurrence or progression. GWAS with larger numbers of patients would also allow for the inclusion of variants with a lower allelic frequency.

If validated, our present findings indicating that three SNPs are associated with aggressive NMIBC, including one also associated with a higher risk of progression, may inform clinicians about the aggressiveness and risk of progression of NMIBC. With an overall price per sample as low as €2.5 (Euros) [30], germline SNP genotyping could be integrated into daily practice at a minimal cost. Additionally, genetic data, in combination with other clinical, and pathology data, may hold future clinical value for the development and application of NMIBC risk prediction models in support of a 'precision prevention' paradigm of targeted disease prevention. In practice, as remarkably shown for breast cancer [8,9], future studies should evaluate the combination of SNP analysis as a polygenic risk score with the current risk prediction model (EORTC or CUETO) to determine whether it adds independent information and improves diagnostic accuracy. Such a diagnosis tool could help better stratify the individual risk of recurrence and progression of NMIBC.

Conclusion

This two-phase study identified three SNPs (rs12615669, rs4976845, and rs2989734) associated with aggressive NMIBC and one SNP (rs4976845) associated with a higher risk of progression. This opens perspectives for future studies to develop and validate a score combining clinical factors (i.e. age, smoking exposure, history of bladder tumours), pathological factors (i.e. tumour Stage and Grade), and genetic factors (i.e. alleles or genotypes of SNPs associated with risk of progression and/or recurrence).

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Conflict of Interest

Morgan Rouprêt is a member of the advisory board of Roche, Ipsen, Astellas, Janssen, GSK, Invecyts, MSD, Arquer, Cepheid, and Nucleix. The remaining authors have nothing to disclose.

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Abbreviations: CIS, carcinoma *in situ*; CUETO, Spanish Urological Club for Oncological Treatment; EORTC, European Organisation for the Research and Treatment of Cancer; FCN1, ficolin 1; GWAS, genome-wide association study; IBCG, International Bladder Consultation Group; IQR, interquartile range; MYO16, myosin XVI; NMIBC, non-muscle-invasive bladder cancer; OR, odds ratio; SNP, single nucleotide polymorphism; TURBT, transurethral resection of bladder tumour; UTUC, upper-urinary tract urothelial carcinoma.