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Inflammatory-Related Genetic Variants in Non-Muscle-Invasive Bladder Cancer Prognosis: A Multimarker Bayesian Assessment

Alexandra Masson-Lecomte[#1,2], **Evangelina López de Maturana**[#1], **Michael E. Goddard**[3,4], **Antoni Picornell**[1], **Marta Rava**[1], **Anna González-Neira**[5], **Mirari Márquez**[1], **Alfredo Carrato**[6], **Adonina Tardon**[7], **Josep Lloreta**[8], **Montserrat Garcia-Closas**[9], **Debra Silverman**[10], **Nathaniel Rothman**[10], **Manolis Kogevinas**[11], **Yves Allory**[12], **Stephen J. Chanock**[10], **Francisco X. Real**[13,14], **Núria Malats**[1], **SBC/EPICURO Study Investigators**

[1]Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain. [2]Urology Department, Henri Mondor Academic Hospital, Paris Est Créteil University, Créteil, France. [3]Biosciences Research Division, Department of Environment and Primary Industries, Agribio, Bundoora, Victoria, Australia. [4]Department of Food and Agricultural Systems, University of Melbourne, Melbourne, Australia. [5]Human Genotyping-CEGEN Unit, Spanish National Cancer Research Centre (CNIO), Madrid, Spain. [6]Servicio de Oncología, Hospital Universitario Ramon y Cajal, Madrid, and Servicio de Oncología, Hospital Universitario de Elche, Elche, Spain. [7]Department of Preventive Medicine, Universidad de Oviedo, Oviedo, Spain. [8]Institut Municipal d'Investigació Mèdica - Hospital del Mar and Departament de Patologia, Hospital del Mar - IMAS, Barcelona, Spain. [9]Division of Genetics and Epidemiology, Institute of Cancer Research, London, United Kingdom. [10]Division of Cancer Epidemiology and Genetics, National Cancer Institute, Department of Health and Human Services, Bethesda, Maryland. [11]Centre for Research in Environmental Epidemiology (CREAL) and Institut Municipal d'Investigació Mèdica – Hospital del Mar, Barcelona, Spain. [12]Pathology Department, Henri Mondor Academic Hospital, Paris Est Créteil University, INSERM, Créteil, France. [13]Epithelial Carcinogenesis Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain.

Corresponding Author: Núria Malats, Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Centre (CNIO), C/Melchor Fernaández Almagro, 3, Madrid 28029, Spain. Phone: 34912246900 (ext. 3330); Fax: 34912246911; nmalats@cnio.es.

Authors' Contributions

Conception and design: A. Masson-Lecomte, E. López de Maturana, M.E. Goddard, N. Rothman, M. Kogevinas, Y. Allory, N. Malats

Development of methodology: A. Masson-Lecomte, E. López de Maturana, M.E. Goddard, A. Tardon, J. Lloreta, N. Malats

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Gonzalez-Neira, M. Márquez, J. Lloreta, M. Garcia-Closas, D. Silverman, N. Rothman, M. Kogevinas, S.J. Chanock, F.X. Real, N. Malats

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Masson-Lecomte, E. López de Maturana, M.E. Goddard, A. Picornell, M. Rava, A. Carrato, A. Tardon, J. Lloreta, F.X. Real, N. Malats

Writing, review, and/or revision of the manuscript: A. Masson-Lecomte, E. Loópez de Maturana, A. Picornell, M. Rava, A. Carrato, A. Tardon, J. Lloreta, M. Garcia-Closas, D. Silverman, N. Rothman, M. Kogevinas, Y. Allory, F. X. Real, N. Malats, N. Malats

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Masson-Lecomte, M. Máarquez

Study supervision: A. Masson-Lecomte, M. Máarquez, N. Malats

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[14]Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain.

[#] These authors contributed equally to this work.

Abstract

Background: Increasing evidence points to the role of tumor immunologic environment on urothelial bladder cancer prognosis. This effect might be partly dependent on the host genetic context. We evaluated the association of SNPs in inflammation- related genes with non-muscle-invasive bladder cancer (NMIBC) risk-of-recurrence and risk-of-progression.

Methods: We considered 822 NMIBC included in the SBC/EPICURO Study followed-up >10 years. We selected 1,679 SNPs belonging to 251 inflammatory genes. The association of SNPs with risk-of-recurrence and risk-of-progression was assessed using Cox regression single-marker (SMM) and multimarker methods (MMM) Bayes A and Bayesian LASSO. Discriminative abilities of the models were calculated using the *c* index and validated with bootstrap cross-validation procedures.

Results: While no SNP was found to be associated with risk-of-recurrence using SMM, three SNPs in *TNIP1, CD5,* and *JAK3* showed very strong association with posterior probabilities >90% using MMM. Regarding risk-of-progression, one SNP in *CD3G* was significantly associated using SMM (HR, 2.69; $P = 1.55 \times 10^{-5}$) and two SNPs in *MASP1* and *AIRE,* showed a posterior probability ≥80% with MMM. Validated discriminative abilities of the models without and with the SNPs were 58.4% versus 60.5% and 72.1% versus 72.8% for risk-of-recurrence and risk-of-progression, respectively.

Conclusions: Using innovative analytic approaches, we demonstrated that SNPs in inflammatory-related genes were associated with NMIBC prognosis and that they improve the discriminative ability of prognostic clinical models for NMIBC.

Impact: This study provides proof of concept for the joint effect of genetic variants in improving the discriminative ability of clinical prognostic models. The approach may be extended to other diseases.

Introduction

Urothelial bladder carcinoma (UBC) is the fifth most common neoplasm in terms of incidence in industrialized countries. UBCis a multifactorial complex disease, tobacco and occupation exposure to aromatic amines being the two best established environmental risk factors (1, 2). In addition, UBC has a genetic component, and candidate gene and genome-wide association studies so far have identified 16 loci associated with UBC risk (3–13)

The majority of UBC are non-muscle-invasive (NMIBC). These tumors are heterogeneous regarding their clinical, pathologic, molecular, and genetic features. Management of NMIBC poses challenges because of their propensity to recur, requiring a long-term surveillance, and their risk to progress to muscle invasion, showing a poor 5-year survival rate (14). The current prognosticators do not completely discriminate between patients who will suffer

from a tumor recurrence/progression and patients who will remain stable after the first transurethral resection of the bladder (TURB); thus justifying the need of prognostic biomarkers to guide the clinical management of patients with NMIBC (15).

Inflammation and cancer are deeply intricate. Not only local inflammation can promote tumor development but also systemic or tumor immune reaction has been shown to have either promoting or opposing cancer effects (16–18). These reactions are however dependent on the host genetic context (19). Previous studies have assessed SNPs involved in inflammatory pathways as prognostic markers for UBC (20–22). Those studies have had limited success, as they have applied simplistic models analyzing each SNP individually, therefore ignoring the complexity of the disease likely underlined by many genetic variants with relatively low effects (23). A recent study has shown the usefulness of multi-marker methods (MMM) able to handle large amount of SNPsç often exceeding the number of individuals, to assess associations between SNPs in inflammatory genes and UBC risk (24).

The objective of this study was to evaluate the association of SNPs in inflammation-related genes with the risk of NMIBC to recur and/or progress by extending the application of MMM to the prognostic field for the first time. We compared results with those coming from the classical single-marker method (SMM) accounting for the time-to-event nature of the data.

Materials and Methods

Ethics statement

Informed consent was obtained from study participants in accordance with the Institutional Review Board of the U.S. National Cancer Institute and the Ethics Committees of each participating hospital.

Study population and tissue samples

We primarily considered the 995 newly diagnosed patients with NMIBC included in the Spanish Bladder Cancer (SBC)/EPICURO Study, a multicenter hospital-based study conducted in 1997–2001 in 18 hospitals (3). Tumors were reviewed and confirmed by trained uropathologists who classified their stage and grade homogeneously using TNM 1997 AJCC and 1973 and 2004 WHO grade classifications. All tumors were transitional cell carcinomas (TCC). Clinical data and information on primary treatment were retrieved from the hospital charts by trained monitors using a structured questionnaire. Patients with NMIBC were classified at high (HiR, $n = 284$) or low (LR, $n = 538$) risk of progression according to the EAU guidelines (25). Low-risk patients consisted of PUNLMP, Ta G1, and G2/low grade and high-risk patients included all T1, G2, and G3/high grade and carcinoma *in situ* (CIS). The intermediate-risk group was not considered herein due to reduced sample size. Patients were followed up for >10 years using both the hospital charts and through direct telephone calls to patients/families. The follow-up rate for patients with NMIBC was 94%.

Gene and SNP selection and genotyping

Germline DNA extracted from blood or saliva, in case blood was not available (4% of the patients), was used for genotyping (3). Genes (n = 251, Supplementary Table S1) were carefully selected according to current available evidence of their involvement in inflammatory processes, favoring those inflammatory genes showing association with cancer as described elsewhere (24). TagSNPs covering these genes were identified using SYSNP (26) and genotyped with the GoldenGate Illumina Genotyping Assay platform (27). On the basis of a literature review, we further included 3,628 SNPs in 52 inflammatory genes already genotyped in the same individuals with the Illumina Infinium HumanHap1M array (6). We excluded SNPs with a low genotyping rate (<95%) and minor allele frequency (MAF) <0.05. Missing genotypes were imputed with BEAGLE (28). To reduce both colinearity between variables and number of statistical tests, pairwise linkage disequilibrium (LD) between SNPs was estimated using the R-package GENETICS (http://cran.r-project.org/web/packages/genetics/index.html). We retained the SNPs with the highest MAF of each LD block when $r^2 > 0.5$. At the end of the quality control process, 822 patients with 1,517 SNP genotypes and complete clinical and pathological information were available for the analysis. These patients were comparable with the whole series (n = 995) for age, gender, area, and tumor stage and grade.

Outcome definition

Time-to-first recurrence (TFR) was defined as time elapsed between first TURB and histologic diagnosis of a new NMIBC of any stage/grade. Time to progression (TP) was defined as time between first TURB and a subsequent histologic diagnosis of a muscle-invasive breast cancer (MIBC), occurrence of metastasis, or death due to bladder cancer.

Statistical analyses

Median follow-up times were obtained by using the reverse Kaplan-Meier method. We applied both an SMM based on a multivariate Cox regression and MMM based on Bayes A (BA) and Bayesian LASSO (BL; Supplementary Fig. S1). Cox proportional hazard regression was used to estimate the HR and 95% confidence intervals (CI) to assess the association between individual SNPs assuming both an additive and a dominant mode of inheritance and the outcomes of interest. Each SNP effect was adjusted for classical clinicopathologic prognosticators for TFR and TP (Supplementary Table S2). TFR analyses did not include patients who received radical cystectomy as first treatment (n = 12). TP was analyzed using all available patients with NMIBC and stratified according to HiR/LR. Stratification was not performed for TFR because survival curves of LRand HiR patients overlapped. Analyses were run in R-language (http://www.R-project.org). SNPs with $P <$ 0.05, 2-sided test, after Bonferroni's correction were kept for comparison with the results from the MMM.

Multimarker methods

Both Bayes A (29) and Bayesian LASSO (30) were applied coupled with a sequential threshold model to analyze each time-to-event data (See Supplementary Methods and ref. 31. This model (32) has been previously used in quantitative genetics settings (33, 34). This

is the first time the method is applied in the prognosis field. The same clinicopathologic adjusting variables were used in the MMM (Supplementary Table S2). Because BA and BL do not provide *P* value, the association strength was estimated using a posterior probability that the SNP is associated with the outcome. An arbitrary threshold of 80% was deemed as significant. Analyses were done using an *ad-hoc* made Fortran program.

Discrimination ability of the model calculation and validation

The discrimination ability of the models including the SNPs showing association with the different outcomes was evaluated by estimating the c index. Briefly, a Cox model including the clinical variables only was compared through the c index with a model including also the previously associated SNPs for each outcome of interest. The *c* index is the frequency of concordant pairs (i.e., the risk of the event predicted by a model is lower for the patient who experiences the event at a later time point) among all pairs of subjects. Three *c* indexes were calculated: the apparent *c* index (calculated with the original data), the bootstrap cross-validation *c* index (calculated using the observations that are not in the bootstrap sample, obtained through a random sampling with replacement), and the bootstrap alone *c* index (i.e., a weighted average of the discrimination in the original dataset and the discrimination in the observations that were not included in the *m*-th bootstrapped sample; as in ref. 35) and R-package 'pec' http://cran.r-project.org/web/packages/pec/pec.pdf).

Results

The median age at diagnosis of the 822 patients with NMIBC was 68 years; 12% of cases were women and 65% of the patients presented LR tumors. Patient and treatment characteristics are displayed in Table 1 and SupplementaryTableS3, respectively. Up to July 2007, median follow-up period for the whole series and for patients "free of disease" were 80.4 and 77.5 months, respectively, with a total of 8 (1.0%) deaths due to UBC as first event. According to the abovementioned definitions, 324 (39.4%) patients suffered, at least, one event. Survival functions for each event are in Supplementary Figs. S2 and S3.

Time to first recurrence

No SNP was found to be associated with TFR by SMM additive model after Bonferroni's correction ($P > 3 \times 10^{-5}$, Supplementary Table S4). Using a dominant mode of inheritance, we found that an SNP in *CARD4/NOD1* (rs10267377) was significantly associated with TRF (HR, 0.58; 95% CI, 0.45–0.75; $P = 0.000026$, $P_{adjusted} = 0.039$). Using MMM, 43 SNPs had PP > 80% of being associated with TFR (Supplementary Table S5). Among them, three had probabilities >90% using both BA and BL, pointing to a very strong association (*TNIP1*-rs2277940, *CD5*-rs7104333, *JAK3*-rs6523, Table 2). The same SNP in *CARD4* had PP of 88% and 90% of being associated with risk of recurrence using BA and BL, respectively.

Time to progression

Only one SNP identified by SMM additive model showed a significant association after Bonferroni's correction: *CD3G*-rs3212262 (HR, 2.69; 95% CI, 1.72–4.23; $P = 1.55 \times 10^{-5}$, $P_{adjusted} = 0.023$; Table 3). Five-year progression-free survival rate was 92% for the AA

genotype versus 84% for the Aa and 71% for the aa (log-rank $P = 0.001$, Fig. 1). No SNP was associated with TP using a dominant model. Using MMM, 2 additional SNPs had PP ≥ 80% with both BA and BL: *MASP1-rs698079* and *AIRE*-rs941405 (Table 4). When assessing only HiR tumors, MMM identified 3 SNPs with PP between 77–80%: *CARD4*-rs2256023, *MAP2K3*-rs9901404, and *TMEM189*-rs2269217 (Supplementary Table S6). Neither SMM nor MMM identified any SNP associated with TP among LR tumors, *CD68*-rs12942088 (Supplementary Table S7) presenting the highest PP (75% with BL). This SNP was one of the top 2 SNPs associated with TP using an additive SMM.

Models' discriminative ability

The clinical parameter model for TFR showed a moderate discriminatory ability (validated *c* index = 0.58; Table 5). By adding the 3 SNPs showing a PP > 90% (*TNIP1*-rs2277940, *CD5*-rs7104333, and *JAK3*-rs6523), the *c* index raised to 0.61. Adding each SNP to the clinical variables increased the predictive ability compared with the model including clinical variables only, showing that the predictive ability of the SNPs does not overlap (see Supplementary Table S8).

The clinical parameter model for TP showed good discrimination ability (validated *c* index = 0.72, Table 5). By adding the 2 SNPs showing PP > 80% (*MASP1*-rs698079 and *AIRE*-rs941405), the *c* index raised to 0.73. The TP predictive ability was also calculated for the HiR and LR subgroups. Adding the SNPs to the clinical variables improved their discrimination ability by 10.5 % for HiR and by 10.2 % for LR in the validation set. As forTFR, the predictive ability of the SNPs did not overlap for TP considering all and HiR patients (see Supplementary Table S8).

Discussion

Classical studies looking for associations between individual SNPs in inflammatory genes and UBC prognosis have had limited success (20–22). While some variants have been previously associated with UBC prognosis (19, 36), significance was in most cases limited to univariate analysis and none of the variant was replicated in independent studies. Moreover, Cox regression is limited by the number of variables the model permits (37). In general, the lack of associations found by SMM outlines its inefficiency to pinpoint variants with small effects in complex traits. To explore the joint effect of multiple SNPs, we have applied MMM strategies mimicking the polygenic scenario that features UBC prognosis. MMM identified, with strong evidence, inflammatory genes with variants individually conferring a small risk of NMIBC recurrence or progression.

A larger number of inflammatory variants showed association with TFR than with TP: 44 SNPs with PP > 80%, 3 of them with PP > 90%, have been associated with TFR. Among them, *JAK3*-rs6523 and CD5-rs7104333 were already identified as associated with UBC risk (24). Only 2 SNPs were associated with TP using MMM. This could be explained by the lower rate of progression events ($n = 76$) compared with the number of recurrences ($n = 268$) that may affect the power of tests in detecting associations. Most of the SNPs/genes associated with TFR were not associated with TP and vice versa, which may indicate that different inflammatory genes trigger distinct NMIBC outcomes. The small correlation

between SNP effects obtained with MMM for TFR and TP (data not shown) also support this hypothesis. However, it is noteworthy that genetic variation in *CARD4/NOD1* was both associated with TR of the whole cohort and TP of high-risk patients. Most of the SNPs identified by MMM also ranked in the first positions when Cox regression was applied. Only two SNPs in *CD3G* and *CARD4* identified by SMM, as associated with TP and TR, passed Bonferroni's correction and their PPs were high: $PP_{BA} = 0.76$ and $PP_{BL} = 0.79$ for *CD3G* and $PP_{BA} = 0.88$ and $PP_{BL} = 0.90$ for *CARD4.* Potential explanations for the different SNP ranking for *CD3G* between tests is the small MAF (0.09) of this variant with very few events in the aa genotype group (Fig. 1; ref. 38) and the adjustment by other SNPs included in the MMMs.

Inflammatory SNPs were not strongly associated with outcome in both HiR and LR subcohorts, probably because of the limited sample size, too. Polymorphisms in inflammatory genes were differently associated with TP in patients at HiR versus LR. Correlation between SMM and MMM estimates of TP in both subcohorts disagreed (Pearson correlation between SNPs effect estimates = −0.01 for BA and −0.03 for Cox regression), this suggesting that the difference in prognosis of both groups may, at least, partially be mediated by inflammatory genes. Risk of TP is highly influenced by Bacillus Calmette-Guérin (BCG) administration (39). Focusing on the HiR subcohort, no BCG*SNP interaction using MMMs was found (results not shown). Larger cohorts might be needed to pinpoint these potential interactions.

SNPs were included as they tagged the selected inflammatory genes, what does not imply a potential function. Since it is difficult to know whether those variants identified are causative or are in high LD with the real ones, they should merely be considered as biomarkers of prognosis. While most of the selected SNPs tagged genes of particular interest in cancer biology, the position of some of the genes changed according to the new version of the human genome release, a fact that misplaced SNPs from the initial selection window (i.e., rs2269217). Noteworthy, most of the significant variants are placed in genes involved in immune tolerance processes: Janus kinase 3 *(JAK3)* participates in intra-cellular signal transduction after activation of immune cells. Lower levels of *JAK3* might be responsible for the defective reactivity of T lymphocytes in patients with cancer (40). *CD5* is known as a negative regulator of T- and B-cell receptor signaling. Its expression has been shown to be implicated in T lymphocytes tolerance toward tumor cells (41). *AIRE* encodes a transcription factor that regulates the expression of tissue antigens in the thymus and plays an important role in the development of organ-specific T regulatory lymphocytes (42). Those T regulators are thought to be major barrier impeding antitumor immune response (42). In melanoma, polymorphisms in *AIRE* may variably affect the selection of melanoma-associated antigen-specific thymocytes, generating T-cell repertoires protecting or predisposing individuals to cancer (43). *CARD4/NOD1* is a member of the NOD receptor family that plays a major role in innate and adaptive immunities. Polymorphisms in those genes have been shown to be associated with multiple cancer risk including UBC (22, 44). *NOD* receptors were demonstrated to be involved in antitumor cytotoxicity through the potentiation of human natural killer cells and macrophage activities (45). *MAP2K3* pathways play a critical role in carcinogenesis. *MAP2K3* has been shown to suppress the growth of breast cancer cells (46) and alterations in its pathway are frequent in UBC (47).

Finally, the protein encoded by *CD3G* is part of the T-cell receptor-CD3 complex. Prognostic impact of T lymphocytes' infiltration is now being investigated in multiple cancers, and *CD3* expression has been shown to be associated with UBC risk of recurrence and mortality (48).

Statistically significant results and potential biologic relevance are not enough criteria to reach clinical utility. Marker(s) have to be clinically actionable and cost efficient. While we identified SNPs strongly associated with NMIBC outcomes, they contributed little (<3.6%) to the higher event prediction ability provided by clinicopathologic variables. Furthermore, these estimates have to be taken cautiously, as their added value might be overestimated mainly because of the relatively low number of progression events in the whole cohort and mainly in the HiR/LR subcohorts. We cannot discard that the performances of the models are still magnified even though their predictive ability was tested by applying bootstrapped cross-validation samples (49). Therefore, an external validation would be advisable to confirm the added predictive value of the identified SNPs. However, heterogeneity across studies regarding patient recruitment, treatment, patient management, or availability of the genotypes data for the same set of SNPs limit the potential success of the replication stage. Furthermore, the definition of inflammatory genes was itself challenging. The list of potential genes is tremendously large, and the edges of the definition are difficult to delineate due to the crosstalk between inflammatory pathways and other cellular functions. It is possible that potential susceptibility markers identified in other studies were not included here, although we estimate these are a minority. Moreover, incomplete mapping of the genes might have occurred as a result of using a previous HapMap genome reference release or filtering by LD, what might have led to missing SNPs of interest. Finally, we did not explore all genetic mechanisms sustaining UBC prognosis, as the genetic architecture and correlations between genes involving complex interactions and epigenetic regulation are still unknown (50).

Despite that, this study reports valuable findings and has noteworthy strengths. The cohort used was built upon strong methodology. All the patients had complete and homogeneously collected clinical, pathologic, and genetic information, with long enough follow-up to investigate NMIBC prognosis. Using innovative MMM that identified many SNPs in inflammatory genes, we provide further evidence of the complex and heterogeneous nature of UBC prognosis and enable to find associations that were not found by applying restrictive SMM.

Conclusion

Considering multiple genetic information jointly is key to understand its influence on complex traits such as UBC outcome. Innovative analytic approaches were essential to demonstrate that several SNPs in inflammatory genes were differently associated with risk of TFR and TP in NMIBC. Although external validation is warranted, this study provides proof of concept for the joint effect of few genetic variants in improving the discriminative ability of clinical prognostic models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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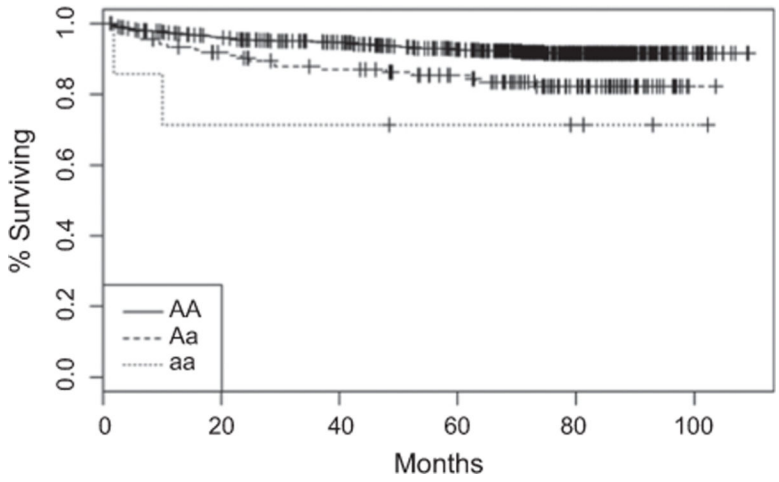

Figure 1.

Progression-free survival of the 822 NMIBC according to *CD3G*-rs3212262 genotypes. Five-year progression-free survival was 92% for AA, 85% for Aa, and 71% for aa genotypes (log-rank $P = 8.4 \times 10^{-4}$, adjusted Cox $P = 0.023$).

Table 1.

Patient and tumor characteristics and number of outcomes according to the NMIBC risk group

	All (N = 822)	Low-risk (*n* = 538)	High-risk (*n* = 284)	*P*
Age, y				7.6×10^{-5}
Median [IQR]	68 (60–73)	67 (58–73)	69 (63–75)	
Mean (SD)	65.5 (10.2)	64.5 (10.8)	67.5 (8.7)	
Gender				0.49
Male	722 (88%)	469 (87%)	253 (89%)	
Female	100 (12%)	69 (13%)	31 (11%)	
TG				-
PUNLMP	41 (5%)	41 (7.6%)	-	
Ta G1	311 (37.8%)	311 (57.8%)	-	
Ta G2	253 (30.8%)	186 (34.6%)	67 (23.6%)	
Ta G3	85 (10.3%)	-	85 (29.9%)	
T1 G2	20 (2.4%)	-	20 (7.1%)	
T1 G3	106 (13%)	-	106 (37.3%)	
Tis G2	1 (0.1%)	-	1 (0.34%)	
Tis G3	5 (0.6%)	-	5 (1.76%)	
Multiplicity				4.2×10^{-5}
≤3 tumors	543 (66%)	383 (71.2%)	160 (56.3%)	
>3 tumors	236 (29%)	130 (24.2%)	106 (37.3%)	
Missing values	43 (5%)	25 (4.6%)	18 (6.4%)	
Size				9.9×10^{-5}
≤3 cm	480 (58.4%)	341 (63.4%)	139 (48.9%)	
>3 cm	112 (13.6%)	58 (10.8%)	54 (19%)	
Missing values	230 (28%)	139 (25.8%)	91 (32.1%)	
Number of patients with tumor recurrence	268 (32.6)	190 (35.3)	78 (27.5)	
Number of patients with progression	76 (9.2)	24 (4.5)	52 (18.3)	

Abbreviation: IQR, interquartile range.

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Table 2.

SNPs with a strong posterior probability (PP > 90%, BA and BL analyses) of being associated with risk of recurrence in NMIBC

			BA		BL		Cox regression	
Gene	**SNP**	**MAF**	**HRaa_AA**	**PP > 90%**	**HRaa_AA**	**PP > 90%**	**HR**	***P***
JAK3	rs6523[a]	0.40	1.69	98	1.17	92	1.20	0.0488
TNIP1	rs2277940	0.07	1.50	91	1.24	94	1.74	0.0001
CDS	rs7104333	0.49	0.72	90	0.86	92	0.81	0.0126

NOTE: The last column displays the adjusted Cox regression results from models including individual SNPs and covariates.

[a]Previously as rs2286662. Analyses were adjusted for geographical area, gender, multiplicity, tumor stage and grade, tumor size, and treatment (see Supplementary Table S2).

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Table 3.

Top 10 autosomal SNPs associated with risk of progression in the whole NMIBC series using multivariable Cox regression additive model

Gene	SNP	HR	*P*	MAF
CD3G	rs3212262	2.70	1.5×10^{-5}	0.09
HLA-B	rs9266462	2.67	0.0026	0.06
CCL-2	rs929259	0.52	0.0081	0.37
FAS	rs1571014	1.73	0.0089	0.36
PPARG	rs7626560	1.85	0.0015	0.19
CXCR4	rs778192	0.55	0.0017	0.38
SOCS5	rs973491	2.11	0.0025	0.07
CXCR4	rs16834018	2.10	0.0035	0.08
CD8B1	rs13024609	1.70	0.0044	0.20
IL6	rs2069827	2.00	0.0053	0.06

NOTE: Analyses were adjusted for geographical area, age, multiplicity, tumor stage and grade, number of recurrences, and treatment (see Supplementary Table S2).

Table 4.

SNPs with strong posterior probability (PP > 80%, BA and BL analyses) of being associated with risk of progression in patients with NMIBC

			BA		BL		Cox regression	
Gene	**SNP**	**MAF**	**HRaa_AA**	**PP > 80%**	**HRaa_AA**	**PP > 80%**	**HR**	***P***
MASP1	rs698079	0.18	1.31	83	1.10	80	1.58	0.0178
AIRE	rs941405	0.37	1.31	86	1.11	83	1.56	0.0116

NOTE: Analyses were adjusted for geographical area, age, multiplicity, tumor stage and grade, number of recurrences, and treatment (see Supplementary Table S2). The last column displays the adjusted Cox regression results from models including individual SNPs and covariates.

Table 5.

Apparent and validated *c* index using Bootstrap alone or 1,000 bootstrap cross-validation indicating the discriminatory ability of the models for each outcome of interest

	Apparent *c* index	**Bootstrap alone *c* index**	**Bootstrap cross-validation *c* index**
Recurrence ALL (*N* = 268 events)			
CV	62.6	60.1	58.4
CV + SNPs (*JAK3, TNIP1, CD5)*	65	62.5	60.5
Progression ALL (*n* = 76 events)			
CV	77.5	74.7	72.1
CV + SNPs (*MASP1, AIRE)*	78.7	75.4	72.8
Progression HiR (*n* = 52 events)			
CV	69.4	63.6	60.1
CV + SNPs *(TMEM189, MAP2K3, CARD4)*	76	70.1	66.4
Progression LR (*n* = 24 events)			
CV	74.2	66.6	62.6
CV + SNPs (*CD68)*	80.8	73.5	69

Abbreviations: CV, clinical variable; *n*, number of events.