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ORIGINAL ARTICLE – CANCER RESEARCH

Altered expression of HER-2 and the mismatch repair genes MLH1 and MSH2 predicts the outcome of T1 high-grade bladder cancer

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Abstract

Purpose The identification of factors predicting the outcome of stage T1 high-grade bladder cancer (BC) is a major clinical issue.

Methods We performed immunohistochemistry to assess the role of human epidermal growth factor receptor-2 (HER-2) and microsatellite instability (MSI) factors MutL homologue 1 (MLH1) and MutS homologue 2 (MSH2) in predicting recurrence and progression of T1 high-grade BCs having undergone transurethral resection of bladder tumor (TURBT) alone or TURBT + intravesical instillations of bacillus Calmette–Guerin (BCG).

Results HER-2 overexpression was a significant predictor of disease-free survival (DFS) in the overall as well as in the two patients' population; as for progression-free survival (PFS), it was significant in the overall but not in the two patients' population. MLH1 was an independent predictor of PFS only in patients treated with BCG and MSH2 failed to predict DFS and PFS in all populations. Most importantly, the higher the number of altered markers the lowers the DFS and PFS. In multivariate Cox proportional-hazards regression analysis, the number of altered molecular markers and BCG treatment were significant predictors ($p = 0.0004$ and 0.0283, respectively) of DFS, whereas the number of altered molecular markers was the only significant predictor ($p = 0.0054$) of PFS.

Conclusions Altered expression of the proto-oncogene HER-2 and the two molecular markers of genetic instability MLH1 and MSH2 predicted T1 high-grade BC outcome with the higher the number of altered markers the lower the DFS and PFS. These findings provide grounds for further testing them in predicting the outcome of this challenging disease.

Keywords Non-muscle-invasive bladder cancer · HER-2 · MLH1 · MSH2 · Immunohistochemistry · Prognosis

Introduction

Bladder cancer (BC) development is a complex multistep process in which oncogenes, tumor suppressor genes as well as genes involved in DNA damage recognition and repair have been implicated (Czerniak et al. 2016).

The DNA damage repair mechanism is crucial to the prevention of changes in nucleic acids by mutagenic risk factors (Peltomäki 2001) and the post-replicative DNA mismatch repair (MMR) system constitutes one of the major DNA-repair pathways in human cells (Peltomäki 2001), resulting in maintenance of genetic stability (Loeb and Loeb 2000). The most important MMR genes are human MutL homologue 1 (hMLH1) and human MutS homologue 2 (hMSH2); their dysfunction may lead to the accumulation of insertion/deletion mutations in simple repeated sequences called microsatellites (MSI

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phenotype) (Dietmaier et al. 1997). Microsatellites residing in oncogenes and tumor-suppressor genes may lead to the development of progressively atypical and malignant cells (O'Brien and Brown 2006). The expression of both hMSH2 and hMLH1 molecules in BC and its correlation with clinical parameters, such as grade, stage and disease outcome, have been studied by both genetic and immunohistochemical methods (Dietmaier et al. 1997; Mylona et al. 2008; Catto et al. 2003; Saetta et al. 2004; Vaish et al. 2005). Results have been quite controversial probably due to the fact that most studies included patients with various tumor stages and grades and having received different treatments.

Human epidermal growth factor receptor-2 (HER-2) is a 185-kDa transmembrane tyrosine kinase receptor; the protein is encoded by ERBB2, a known proto-oncogene located at the long arm of human chromosome 17 (17q12). It is involved in oncogenesis via activation of intracellular pathways leading to proliferation, angiogenesis, cell survival, and metastatic potential (Laé et al. 2010). As for the MMR proteins MLH1 and MSH2, the role of HER-2 expression in BC is controversial (Lammers and Witjes 2011; Chen et al. 2013), mainly due to heterogeneity of tested populations.

In the last decade, efforts have been made to test molecular markers in BC populations homogeneous for tumor stage and grade as well as for given treatment; specific attention has been given to T1 high-grade BC, as its unpredictable behaviour makes the identification of reliable prognostic factors of disease outcome a real clinical priority. Markers such as p53, pRb, p21, and survivin have proved to reliably predict the outcome of T1 high-grade BC treated by complete trans-urethral resection of bladder tumor (TURBT) and intravesical instillation of BCG; therefore, they seem to be ready for clinical use (Sanguedolce et al. 2014). On the other hand, novel molecules are emerging not only as potential prognostic/predictive markers but also as potential therapeutic targets and, therefore, await validation in homogeneous patients' populations (Sanguedolce et al. 2015). Finally, recent studies suggest that the higher the number of altered molecular markers the greater the risk of non-muscle-invasive bladder cancer (NMIBC) recurrence and progression (Shariat et al. 2007; Cormio et al. 2009). Both studies, however, tested a single pathway of BC carcinogenesis (cell cycle regulators).

In the present study we tested, in a homogeneous population of T1 high-grade BCs, two different pathways of carcinogenesis; we chose the proto-oncogene HER-2, as we recently found it to be a significant predictor of high-grade T1 BC outcome (Cormio et al. 2017a, b), and MLH1 and MSH2, as we theorized that simultaneous genetic instability might increase the negative predictive value of HER-2 overexpression.

Materials and methods

Study population consisted of patients who underwent complete TURBT from January 2005 to September 2012 and were diagnosed with T1 high-grade BC by a single uropathologist (FS). Inclusion criteria were: (1) bladder muscle clearly identifiable and free of disease; (2) negative restaging TURBT (including random bladder biopsies) within 4 months after the first TURBT; (3) complete follow-up data. Exclusion criteria were: (1) presence of concomitant carcinoma in situ and/or variant histology; (2) shift from no adjuvant treatment to adjuvant BCG during follow-up; (3) incomplete BCG treatment. Patients who did not receive BCG treatment actually refused it; "complete" BCG treatment included induction with one intravesical instillation (Pasteur strain, 75 mg in 50 ml saline) once a week for 6 consecutive weeks, followed by maintenance (one instillation every 3 months for 1 year).

Follow-up consisted of urine cytology and cystoscopy every 3 months for the first 2 years, every 6 months for the third year, and then yearly. Abdominal computed tomography was performed at initial diagnosis and then every second year to rule out upper tract disease. Tumor recurrence was defined as pathological evidence of disease at bladder biopsy or TURBT, whereas tumor progression was defined as pathological shift to muscle-invasive disease at bladder biopsy or TURBT or imaging techniques demonstrating recurrent bladder cancer and distant metastasis likely correlated to it. The study was approved by the Internal Review Board.

Immunohistochemical staining

Serial section 4 μm thick were cut from formalin-fixed paraffin-embedded tissue, deparaffinized in xylene, rehydrated in graded ethanol solutions, washed for 5 min with distilled water and mounted on poly-L-lysine-coated glass slides.

MLH1, MSH2 and HER-2 expression was assessed by standard linked streptavidin–biotin horseradish peroxidase technique using specific monoclonal antibodies against MLH1 (mouse monoclonal primary antibody, clone M1), MSH2 (mouse monoclonal primary antibody, clone G219–1129), and HER-2 (rabbit monoclonal primary antibody, clone 4B5, PATHWAY) delivered by the Benchmark XT autostainer (Ventana Medical Systems Inc, Tucson, AZ). Positive and negative controls were used. For each specimen, the whole section was examined under light microscopy (400× magnification), and the immunohistochemical expression was assessed as follows:

- MLH1 and MSH2: the number of positive nuclei was manually counted in five areas and then assessed as a

percentage (labeling index). Staining for both proteins was scored as positive (preserved expression) or negative (reduced expression) according to the percentage of nuclear immune labelling and the staining intensity; cases displaying less than 20% positive tumor cells and/or very faint staining were classified as negative (MSI phenotype) (Saetta et al. 2004) (Fig. 1a, b).

- HER-2: a four-point scale was used: '0' if there was no membranous staining; '1 +' if there was weak membranous staining in at least 10% of cells; '2 +' if there was moderate membrane staining in at least 10% of cells; and '3 +' if there was strong membranous staining in at least 10% of cells. Scores 2 + and 3 + were considered positive while scores 0 and 1 + were considered negative (Olsson et al. 2012; Ding et al. 2015) (Fig. 1c).

All cases were independently reviewed by another senior pathologist (PB) unaware of clinical data and the original diagnosis; he also reviewed agreement with the latest WHO Classification of Tumors of the Urinary System and Male Genital Organs (Moch et al. 2016) and the 2010 TNM staging system (Edge et al. 2010).

Statistical analysis

Univariate survival analysis was carried out using the Kaplan–Meier method, with differences among groups being tested for significance using the log-rank test. Multivariate analysis of probable prognostic factors for survival was performed with Cox's proportional hazard regression analysis. Differences in rates were tested with the Fisher's exact test, whereas differences between continuous variable were tested with the Student's *t* test. Statistical analysis was carried out using the STATA SE 14 (StataCorp, College Station, Texas, USA). Significance was set at $p < 0.05$.

Results

A total of 67 patients fulfilled the study inclusion and exclusion criteria; their mean age was 71.7 ± 9.89 years. As expected, patients who underwent BCG treatment were younger than those who did not (67.9 ± 10.56 vs. 75.3 ± 7.84 years, respectively; $p = 0.002$). Patients' characteristics and the treatment they received are summarized in Table 1.

At median follow-up of 75.7 months (range 9–133), recurrent NMIBCs were found in 35 patients; of them, 8 experienced subsequent disease progression (7 local and 1 associated to liver metastases). Conversely, nine patients experienced direct disease progression (eight local and one associated to multiple pulmonary metastases). Thirteen patients underwent cystectomy, four because of recurrent

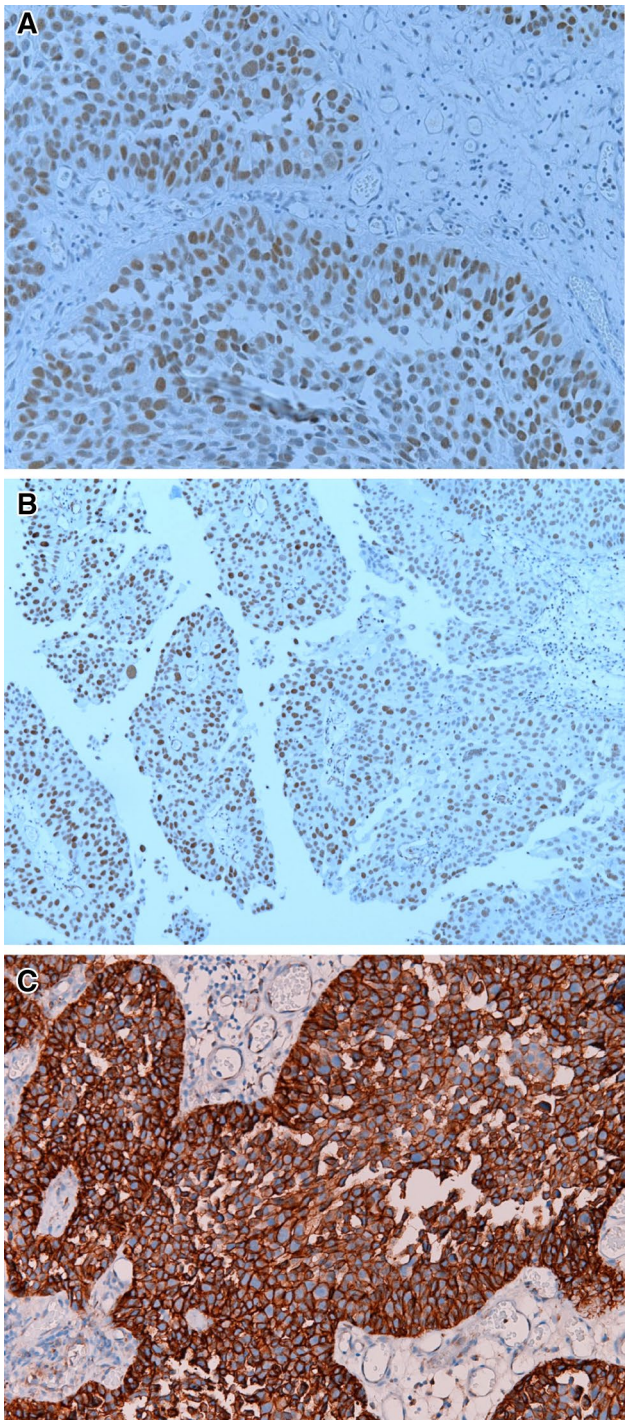

Fig. 1 Representative images of MLH1 (**a**), MSH2 (**b**) and HER2 (**c**) immunohistochemical expression of T1 high-grade bladder cancers exhibiting strong and diffuse staining are shown

T1 high-grade disease and nine because of local disease progression; the former were excluded from progression-free survival evaluation. Fourteen patients eventually died, ten from their BC and four from other causes. Therefore, the overall disease-free, progression-free and

Table 1 Patients' demographic and pathologic characteristics

Variable	Overall 67 pts	BCG 34 pts	No BCG 33 pts	p value
Male	59	30	29	1.000
Female	8	4	4	
Primary	59	30	29	1.000
Secondary	8	4	4	
Single	49	24	25	0.784
Multiple	18	10	8	
Size < 3 cm	31	17	14	0.628
Size > 3 cm	36	17	19	
HER-2 negative	38	18	20	0.624
HER-2 positive	29	16	13	
MLH1 negative	33	16	17	0.8086
MLH1 positive	34	18	16	
MSH2 negative	45	20	25	0.1944
MSH2 positive	22	14	8	

cancer-specific survival rates were 35.8, 73.0 and 85.1%, respectively.

Kaplan–Meier estimators and log-rank test (Table 2) showed that "traditional" prognostic factors failed to predict DFS and PFS in patients treated and not-treated with BCG. The role of HER-2, MLH1 and MSH2 in predicting DFS and PFS in the overall population as well as in the two treatments groups is summarized in Table 2.

The finding that patients treated and not-treated with BCG has similar number and characteristics (Table 1) allowed to evaluate the effect of BCG treatment on disease outcome. The disease-free rate was 47.1% (16/34) in patients who received (mean follow-up 85.29 months) and 24.2% (8/33) in patients who did not received BCG treatment (mean follow-up 65.33 months); the difference in DFS (Fig. 2a) was statistical significant ($p = 0.0231$). The progression-free rate was 69.7% (10/33) in patients who received and 76.7% (7/30) in patients who did not received BCG treatment; the difference in PFS (Fig. 2b) was not statistically significant ($p = 0.6901$).

Table 2 Univariate survival analysis according to Kaplan–Meier method and the log-rank test

Variable	Disease-free survival			Progression-free survival		
	Overall 67	BCG 34 pts	No BCG 33 pts	Overall 63	BCG 33 pts	No BCG 30 pts
Male vs. female	0.3400	0.3430	0.5647	0.3355	0.1887	0.9311
Primary vs. recurrent	0.1926	0.5363	0.1614	0.6409	0.0519	0.2591
Single vs. multiple	0.4756	0.7956	0.2035	0.6412	0.8857	0.3905
Size < 3 cm vs. >3 cm	0.3926	0.2524	0.7226	0.7417	0.2783	0.4515
HER-2 negative vs. HER-2 positive	**0.0013**	**0.0140**	**0.0125**	**0.0322**	0.1290	0.1696
MLH1 negative vs. positive	0.3348	0.1116	0.6114	0.1482	**0.0123**	0.6279
MSH2 negative vs. positive	0.1962	0.2711	0.8672	0.6236	0.2465	0.6690

Bold value indicates a statistically significant difference with a *p*-value less than 0.05

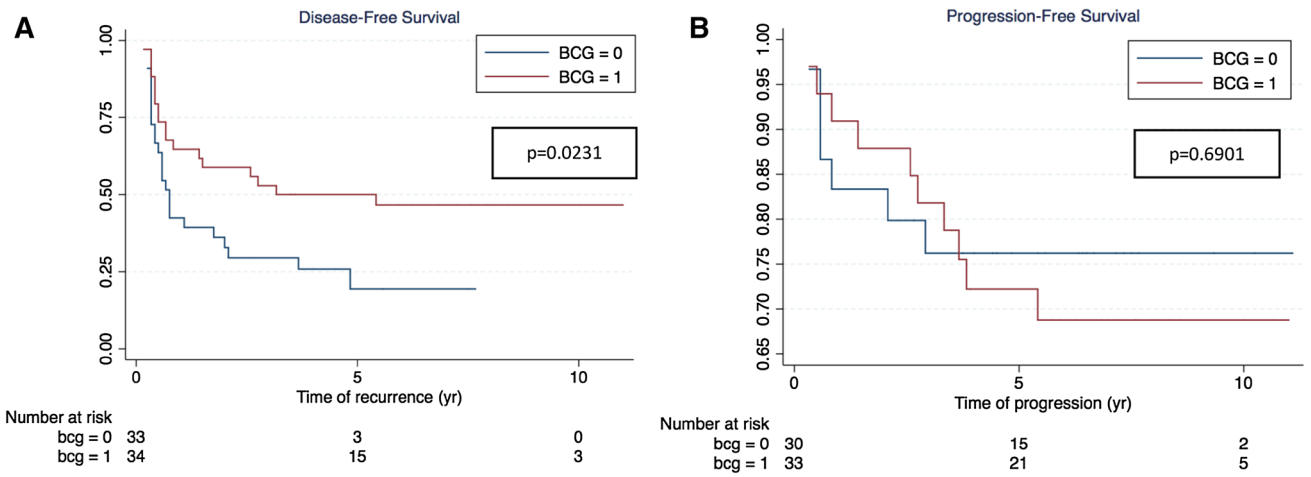

Fig. 2 Kaplan–Meier curves of disease-free (**a**) and progression-free survival (**b**) in patients treated (1) or not (0) with BCG

Finally, we tested the ability of number of altered markers in predicting DFS and PFS, scoring one altered expression of only one MMR marker (either MLH1 or MSH2), two altered expression of either HER-2 or the two MMR markers, three altered expression of both HER-2 and one of the two MMR markers, and 4 altered expression of HER-2 and both MMR markers. Disease-free rate was 100, 36.4, 32.1, 27.3 and 0%, for 0, 1, 2, 3, and 4 altered molecular markers, respectively; the difference in DFS (Fig. 3a) was statistically significant ($p=0.0016$). Progression-free rate was 100, 80, 73.1, 70 and 44.4%, for 0, 1, 2, 3, and 4 altered molecular markers, respectively; the difference in PFS (Fig. 3b) was statistically significant ($p=0.0369$). Multivariate Cox proportional-hazards regression analysis pointed out that the number of altered molecular markers and BCG treatment were significant predictors ($p=0.000$ and $p=0.029$, respectively) of DFS, whereas the number of altered molecular markers was the only significant predictor ($p=0.008$) of PFS (Table 3; Fig. 4).

Discussion

To date, prediction of recurrence and progression of NMIBC relies on the EORTC and the Club Urologico Espanol de Tratamiento Oncologico (Spanish Urological Oncology Group, CUETO) scoring systems (Babjuk et al. 2017). Unfortunately, both suffer the bias of being based on patients with different tumor stage and grade and/or having received different treatment. It is, therefore, not surprising that "traditional" factors such as tumor size, tumor number, tumor recurrence and even concomitant carcinoma in situ failed to predict recurrence and progression when they were tested in homogeneous population of T1 high-grade BCs treated with BCG (Palou et al. 2012; Sanguedolce et al. 2015). Accordingly, current EAU guidelines on NMIBC (Babjuk et al. 2017) acknowledge that "research is needed to determine the role of molecular markers in improving the predictive accuracy of currently available risk tables".

The present study confirmed that "traditional" prognostic factors on which the EORTC and CUETO risk calculators are based failed to predict recurrence and progression of T1 high-grade BC, treated or not with BCG. HER-2 overexpression proved to be a significant predictor of DFS in the overall as well as in the two patients' population, and of PFS in the overall but not in the two patients' population. MLH1 was an independent predictor of PFS only in patients treated with BCG and MSH2 failed to predict DFS and PFS in all populations.

As mentioned above, the role of MLH1 and MSH2 in predicting BC outcome is controversial. Jin et al. (1999) found, in 115 cases of BC of all stage and grade, that tumors

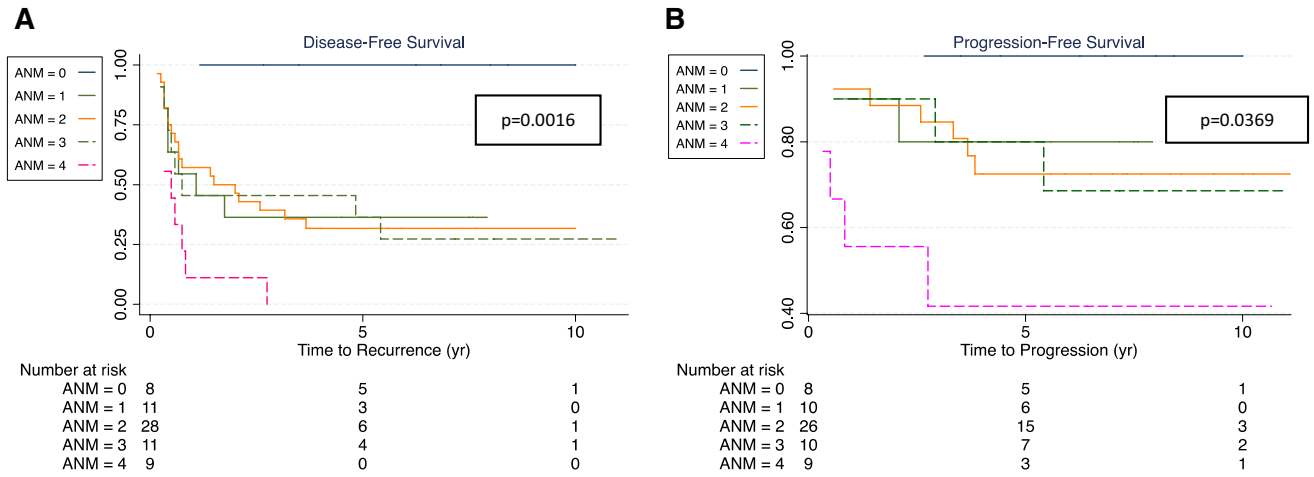

Fig. 3 Kaplan–Meier curves of disease-free (**a**) and progression-free (**b**) survival according to number of altered molecular markers (#AMM)

Table 3 Multivariate Cox proportional-hazards regression analysis

Variable	Disease-free survival			Progression-free survival		
	RR	95% CI of HR	*p* value	RR	95% CI of HR	*p* value
BCG treatment	0.5040	0.2722–0.9334	**0.029**	1.2360	0.4703–3.2485	0.667
#AMM	1.6707	1.2549–2.2242	**0.000**	1.8499	1.1753–2.9119	**0.008**

Bold value indicates a statistically significant difference with a *p*-value less than 0.05

AMM number of altered molecular markers, *HR* hazard rato

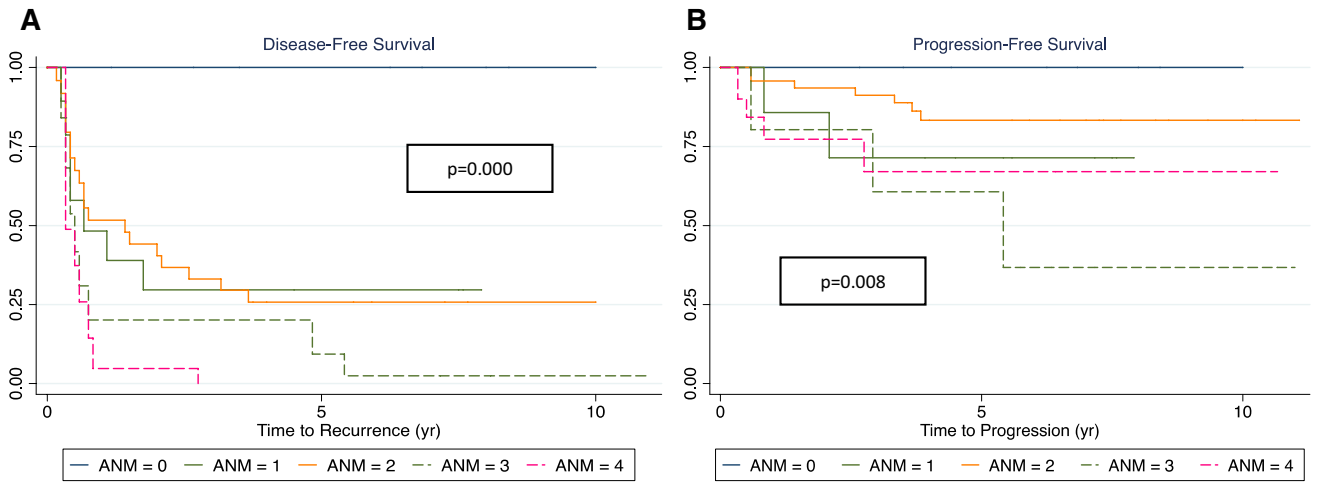

Fig. 4 Multivariate Cox proportional-hazards regression analysis of disease-free (**a**) and progression-free (**b**) survival probability according to number of altered molecular markers (#AMM)

with reduced hMSH2 expression were mostly high-grade/high-stage, and displayed significantly higher relapse rates compared to those with normal expression. Similarly, Saetta et al. (2004) showed, in 72 cases of primary BC of all grades and stages, that reduced hMLH1 expression was a significant predictor of shorter DFS. Finally, Catto et al. (2003) tested 111 BCs of all stage and grade and found that reduced expression was seen more commonly in muscle invasive and high-grade than in superficial, low-grade tumors; on the other hand, reduced expression of either MMR protein was associated, by 5 years, with fewer recurrences of superficial tumors and fewer relapses in all tumors compared to tumors with normal expression.

Also the role of HER-2 overexpression in predicting BC outcome remains controversial. Chen et al. (2013) reported that a subset of high-grade NMIBCs contained HER-2 amplification and was associated with markedly aggressive behavior; similarly, Ding et al. (2015) demonstrated that HER-2 overexpression was a significant predictor of progression, especially in patients with intermediate- and high-risk EORTC scores. On the other hand, Olsson et al. (2012) reported no significant association between HER-2 status and prognosis in 285 patients with primary T1 BC. Again, all these studies were biased by heterogeneity of tumor stage and grade, as well as of given treatment. Bongiovanni et al. (2013) tested the prognostic role of HER-2 expression in 83 patients with T1 high-grade BC and found that this marker was not a significant predictor or tumor recurrence or progression; however, it is not clear whether or not these patients received adjuvant BCG treatment after TURBT and, in any case, none underwent restaging TUR at any stage. Conversely, we recently demonstrated that, in a homogeneous population of patients with T1 high-grade BC, HER-2 expression was the most significant predictor of DFS and PFS, performing better than "traditional" prognostic factors as well as of BCG treatment (Cormio et al. 2017a, b).

The strength of our study is being the first testing the role of MLH1 and MSH2 in a homogeneous population of patients with T1 high-grade BC having undergone a well-defined treatment (TUR alone vs. TUR + BCG induction and 1 year maintenance) and having had a negative restaging TURBT. This careful patients' selection should guarantee for reliability of obtained results. The other strong point of our study is having tested combined expression of these novel markers (MLH-1 and MSH-2) with HER-2 expression in predicting the outcome of this disease. Findings were clear: the higher the number of altered biomarkers the greater the risk of disease recurrence and progression, with rates ranging from 0% when no marker was altered to 100% when all three markers were altered. Such findings are in agreement with a previous study whereby Shariat et al. (2007) demonstrated that the combination of several cell cycle markers (p53, pRb, p21 and p27) had cooperative/synergistic effects in stratifying patients with NMIBC into different risk groups; specifically, the higher the number of altered biomarkers the greater the risk of disease recurrence and progression. However, these authors tested a single pathway of BC carcinogenesis (cell cycle regulators) in 74 patients with NMIBC of different stage and grade; conversely, we tested two different pathways of carcinogenesis, namely genetic instability and the proto-oncogene HER-2, in a homogeneous population of T1 high-grade BCs. In line with these studies, we previously demonstrated, in a homogeneous population of T1 high-grade BCs treated with BCG, that altered expression of two cell cycle regulators (p53 and pRb) was associated with a greater risk of recurrence than altered expression of a single such marker (Cormio et al. 2009, 2010); most important, progression rate was 50%

when both markers were altered as compared to 0% when one or no marker was altered.

Potential limitations of our study include its retrospective nature, but this applies to all studies presently available, and its relatively small sample size, but we believe that a well- selected and homogeneous population provides more valuable information than a larger but not homogeneous one. Finally, our study did not provide information on potential benefit of "aggressive" treatment, i.e. early radical cystectomy, in patients with T1 high-grade BC and combined molecular markers overexpression but this was not our policy in such tumors.

In conclusion, reliable molecular signatures of BC are eagerly awaited to predict outcome of available treatments and, possibly, to identify novel more effective targeted treatments (Cormio et al. 2017a, b). The present study first demonstrated that, in patients with T1 high-grade BC having undergone "conservative treatment", altered expression of the proto-oncogene HER-2 and the two molecular markers of genetic instability MLH-1 and MSH-2 predicted disease outcome with the higher the number of altered markers the lower the DFS and PFS. BCG treatment significantly reduced recurrence but not progression; overall, tumor biological features appeared to be more relevant than BCG treatment in determining disease outcome.

Compliance with ethical standards

Conflict of interest Francesca Sanguedolce declares that she has no conflict of interest. Antonella Cormio declares that she has no conflict of interest. Paolo Massenio declares that he has no conflict of interest. Maria C. Pedicillo declares that she has no conflict of interest. Simona Cagiano declares that she has no conflict of interest. Francesca Fortunato declares that she has no conflict of interest. Beppe Calò declares that he has no conflict of interest. Giuseppe Di Fino declares that he has no conflict of interest. Giuseppe Carrieri declares that he has no conflict of interest. Pantaleo Bufo declares that he has no conflict of interest. Luigi Cormio declares that he has no conflict of interest.

Ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Human and animal rights statement This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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