miR-199a-3p and miR-214-3p improve the overall survival prediction of muscle-invasive bladder cancer patients after radical cystectomy

Thorsten H. Ecke¹, Katja Stier², Sabine Weickmann³, Zhongwei Zhao³, Laura Buckendahl³, Carsten Stephan³,⁴, Ergin Kilic⁵ & Klaus Jung³,⁴

¹Department of Urology, HELIOS Hospital, Bad Saarow, Germany
²Department of Urology, Campus Benjamin Franklin, University Hospital Charité, Germany
³Department of Urology, Campus Charité Mitte, University Hospital Charité, Germany
⁴Berlin Institute for Urologic Research, Berlin, Germany
⁵Institute of Pathology, University Hospital Charité, Germany

© 2017 The Authors. Cancer Medicine published by John Wiley & Sons Ltd.
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Abstract
To improve the clinical decision-making regarding further treatment management and follow-up scheduling for patients with muscle-invasive bladder cancer (MIBC) after radical cystectomy (RC), a better prediction accuracy of prognosis for these patients is urgently needed. The objective of this study was to evaluate the validity of differentially expressed microRNAs (miRNAs) based on a previous study as prognostic markers for overall survival (OS) after RC in models combined with clinicopathological data. The expression of six miRNAs (miR-100-5p, miR-130b-3p, miR-141-3p, miR-199a-3p, miR-205-5p, and miR-214-3p) was measured by RT-qPCR in formalin-fixed, paraffin-embedded tissue samples from 156 MIBC patients who received RC in three urological centers. Samples from 2000 to 2013 were used according to their tissue availability, with follow-up until June 2016. The patient cohort was randomly divided into a training (n = 100) and test set (n = 56). Seventy-three samples from adjacent normal tissue were used as controls. Kaplan–Meier, univariate and multivariate Cox regression, and decision curve analyses were carried out to assess the association of clinicopathological variables and miRNAs to OS. Both increased (miR-130b-3p and miR-141-3p) and reduced (miR-100-5p, miR-199a-3p, and miR-214-3p) miRNA expressions were found in MIBC samples in comparison to nonmalignant tissue samples (P < 0.0001). miR-199a-3p and miR-214-3p were independent markers of OS in Cox regression models with the significant clinicopathological variables age, tumor status, and lymph node status. The prediction model with the clinicopathological variables was improved by these two miRNAs in both sets. The predictive benefit was confirmed by decision curve analysis. In conclusion, the inclusion of both miRNAs into models based on clinical data for the outcome prediction of MIBC patients after RC could be a valuable approach to improve prognostic accuracy.

Introduction
Bladder cancer is the fifth most frequent cancer in Europe. In 2012, its incidence and annual mortality rate were estimated to 151,200 and 51,400 cases, respectively [1]. Approximately 30% of these patients suffered from muscle-invasive bladder cancer (MIBC) at the time of initial diagnosis [2]. Radical cystectomy (RC) is the gold standard to treat these patients. In contrast to patients with nonmuscle-invasive bladder cancer (NMIBC), MIBC patients are subject to a high risk of relapse following RC and cancer-related death.
In order to remedy this unsatisfactory situation, serious efforts have recently focused on new therapeutic strategies regarding the application of neoadjuvant and adjuvant chemotherapies [3]. A better risk assessment of patients has been recommended by developing novel predictive/prognostic models [4]. In clinical practice, the therapeutic management of these patients has so far been performed almost exclusively on the basis of clinical data and classical pathological TNM criteria but with few reliable results [4]. There is the hope that the identification of new molecular tissue biomarkers could help to stratify risk groups and determine which patients benefit from adjuvant strategies after surgery [5]. While the results of numerous studies using immunohistochemical biomarkers have been rather disappointing regarding their clinical utilization [6], recent reports on gene-based approaches [5, 7, 8], including using microRNAs (miRNAs) as a new class of mRNA regulators seem to be much more promising [9–17].

In a previous study, we identified numerous new differentially expressed miRNAs in fresh-frozen bladder cancer tissue samples in comparison to normal adjacent tissue [18]. It was therefore the objective of this study to evaluate from these 15 previously described miRNAs the usefulness of the most promising miRNAs regarding their potential predictive ability of overall survival (OS) and assess their benefit in comparison to conventional clinicopathological variables in MIBC patients after RC. Criteria for the selection of these miRNAs in this study were determinations in a well measurable Cq range and at least twofold median expression differences between the nonmalignant and MIBC tissue samples to allow robust measurements as described before [18]. According to these criteria, both three up-regulated (miR-130b-3p, miR-141-3p, and miR-205-5p) and three down-regulated (miR-100-5p, miR-199a-3p, and miR-214-3p) miRNAs were included in this study. To simulate the conditions of clinical practice formalin-fixed, paraffin-embedded (FFPE) tissue samples from three urological centers were analyzed corresponding to a multi-center study.

**Materials and Methods**

**Patients and tissue samples**

This study included 156 MIBC patients without any neoadjuvant therapy who underwent RC at three urological centers from 2000 to 2013. Sixty-four patients (41%) received adjuvant cisplatin-based chemotherapy after RC (Table 1). Follow-up data collected until June 2016 were based on medical records and telephone contact with the patients’ urologists and patients or family members and death certificates. The study was approved by the Hospital Ethics Committee (EA1/153/07; EA1/134/12) in accordance with the Declaration of Helsinki. The new STARD and REMARK guidelines were considered [19, 20].

The study was carried out on FFPE tissue specimens selected according to their tissue availability and follow-up data (Table 1). All samples were finally reviewed by an expert uropathologist (EK) according to the criteria of the International Union Against Cancer and the World Health Organization/International Society of Urological Pathology, as previously reported [18].

### RNA extraction and quantitative RT-PCR of miRNAs

Previously described analytical procedures were applied [18, 21]. Hematoxylin/eosin staining was used to identify tumor areas with >80% tumor cells and nonmalignant areas. The areas of interest were punch-biopsied with a 1-mm needle device and extracted with the miRNeasy FFPE Kit with an additional DNase I digestion step (Qiagen, Hilden, Germany). RT-qPCR measurements and normalizations were performed for the quantification of the miRNAs with TaqMan miRNA assays (Applied Biosystems, Foster City; Table S1) on a Light Cycler 480 Instrument (Roche Diagnostics, Mannheim, Germany) as previously described including the related documentation with regard to the specific items of the MIQE guidelines [18, 21, 22].

### Statistical analysis

QBasePLUS, v.3.0 software (Biogazelle, Zwijnaarde, Belgium) was used for the analysis of RT-qPCR data. Statistical analyses were performed with SPSS 23 (SPSS Inc., Chicago) and MedCalc 16.8.4 (MedCalc Software, Ostend, Belgium). Nonparametric statistical tests (Spearman rank correlation, Mann-Whitney U test, Kruskal–Wallis test) were used. The discriminating capacity of miRNAs was assessed by receiver operating characteristics (ROC) analysis and binary logistic regression. Kaplan–Meier and Cox regression analyses were performed for OS. Decision curve analysis and time-dependent ROC analysis using the statistical approach of cumulative case/dynamic control and incident case/dynamic control ROC analysis were used to assess the predictive benefit and accuracy of the examined markers [23, 24]. The SPSS bias-corrected and accelerated bootstrap method was used for the internal validation using the patient cohort by splitting at random into a training (n = 100) and test set (n = 56). P-values <0.05 (two-tailed) were considered statistically significant.
Results

Study design and patient characteristics

This retrospective study from three urologic departments included 156 MIBC patients that were randomly divided into a training ($n = 100$) and test set ($n = 56$). For comparison purposes, 73 nonmalignant tissue samples available from these patients were used as controls. The characteristics of the study groups are summarized in Table 1. There were no statistically significant differences in age and sex between the controls and MIBC patients and between the training and test set with regard to the important clinicopathological variables (Table 1).

Differential expression of microRNAs in bladder tissue of MIBC patients

The expression data of the six miRNAs of interest (miR-100-5p, miR-130b-3p, miR-141-3p, miR-199a-3p, miR-205-5p, miR-214-3p) were not statistically different ($P = 0.084–0.792$) between the three centers, so merged data could be evaluated. Figure 1 shows that the miRNA expressions differed with high statistical significance ($P < 0.0001$) between nonmalignant and MIBC samples except miR-205-5p. Both increased (miR-130b-3p, miR-141-3p) and reduced levels (miR-100-5p, miR-199a-3p, miR-214-3p) were found in MIBC samples in comparison to nonmalignant tissue samples ($P < 0.0001$). The differential miRNA expressions corresponded to the capacity of the miRNAs to discriminate between nonmalignant and MIBC tissue with miR-130b-3p as the best discriminator (Table S2 and Fig. S1A and S1B).

Correlation of miRNA expressions to clinicopathological variables and among each other

Correlations were not observed between all miRNAs and sex or age in nonmalignant and tumor samples ($r_s = 0.108–0.001$, $P$-values of 0.180–0.915). The clinicopathological

| Variable                              | Controls ($n = 73$) | All MIBC patients ($n = 156$) | $P^2$ | Training set ($n = 100$) | Test set ($n = 56$) | $P^2$ |
|---------------------------------------|---------------------|--------------------------------|-------|--------------------------|---------------------|-------|
| Age [year, median (range)]            | 69 (44–81)          | 69 (37–82)                    | 0.689 | 69 (37-82)               | 68 (45–81)          | 0.283 |
| Gender ([n; %])                       |                     |                                |       |                          |                     |       |
| Female                                | 7 (10)              | 23 (15)                       | 0.401 | 12 (12)                  | 11 (20)             | 0.241 |
| Male                                  | 66 (90)             | 133 (85)                      |       | 88 (88)                  | 45 (80)             |       |
| pT status ([n; %])                    |                     |                                |       |                          |                     |       |
| pT2                                   | 47 (30)             | 32                             |       |                          |                     | 0.759 |
| pT3                                   | 78 (50)             | 48                             |       |                          |                     |       |
| pT4                                   | 31 (20)             | 20                             |       |                          |                     |       |
| Grade ([n; %])                        |                     |                                |       |                          |                     |       |
| G2                                    | 14 (9)              | 10                             |       |                          |                     | 0.463 |
| G3                                    | 140 (90)            | 88                             |       |                          |                     |       |
| G4                                    | 2 (1)               | 2                              |       |                          |                     |       |
| pN status ([n; %])                    |                     |                                |       |                          |                     |       |
| pN0/Nx                                | 102 (65)            | 69                             |       |                          |                     | 0.222 |
| pN1                                   | 54 (35)             | 31                             |       |                          |                     |       |
| Adjuvant therapy ([n; %])             |                     |                                |       |                          |                     |       |
| Yes                                   | 64 (41)             | 43                             |       |                          |                     | 0.611 |
| No                                    | 92 (59)             | 57                             |       |                          |                     |       |
| Sample source ([n; %])                |                     |                                |       |                          |                     |       |
| Center 1                              | 51 (70)             | 101 (65)                      | 0.486 | 62                       | 39                  | 0.598 |
| Center 2                              | 7 (10)              | 24 (15)                       |       | 16                       | 8                  |       |
| Center 3                              | 15 (20)             | 31 (20)                       |       | 22                       | 9                  |       |
| Follow-up after surgery               |                     |                                |       |                          |                     |       |
| Time [month; median (range)]          | 28 (1–180)          | 34 (1–180)                    |       | 23 (1–163)               |                     | 0.503 |
| Death events ([n; %])                 | 99 (63)             | 67 (67)                       |       | 32 (57)                  |                     | 0.230 |
| Survival time [month, median (95% CI)]| 34 (24–49)          | 35 (24–49)                    |       | 26 (16–106)              |                     | 0.589 |

CI, confidence interval; G, histopathological grade; MIBC, muscle-invasive bladder cancer; pN, lymph node status; pT, pathological tumor classification.

1Controls refer to nonmalignant tissue samples obtained from MIBC patients as described in Methods and Materials.

2Statistical tests: Mann–Whitney U test; Chi-square or Fisher’s exact test, and log-rank test, Kaplan–Meier analysis.

3Center 1: Campus Benjamin Franklin, University Hospital Charité; Center 2: Campus Mitte, University Hospital Charité; Center 3: Helios Clinical Center, Bad Saarow.
variables pT status, histopathological grade, and lymph node status were also not significantly associated with all miRNAs ($r_s = 0.113–0.004$, $P$-values of 0.160–0.957) except for miR-130b-3p and miR-205-5p to grade ($r_s = 0.236$ to $-0.197$, $P$-values of 0.003 and 0.014). These missing associations of miRNAs to clinicopathological variables corresponded to results obtained by the Kruskal–Wallis (pT status, histological grade) and Mann–Whitney test (lymph node status). Different correlation coefficients between miRNAs were observed between nonmalignant and MIBC tissue (Table S3).

**Prognostic potential of miRNAs predicting postcystectomy overall survival of MIBC patients**

The differential expression and the different correlations between the examined miRNAs indicate their potential as prognostic markers. To substantiate the relationship between the clinical outcome of patients and the clinicopathological variables as well as miRNAs, OS was analyzed as the primary clinical endpoint since reliable information about cancer death was not available in all cases. OS was
defined as the time from the date of RC until the time of death or the last follow-up.

Kaplan–Meier survival analyses of clinicopathological variables showed a decreased OS depending on the lymph node metastasis, increased pT status, and age, but OS was not associated with adjuvant chemotherapy (Fig. S2). These initial data proved the representativeness of the study cohort to evaluate the impact of miRNAs regarding their usefulness as prognostic markers. For the internal validation of data, the prognostic performance of all clinicopathological variables and the six miRNAs was calculated using the training and test set (Table 2). The variables with \( P \)-values <0.10 in univariable analyses for clinicopathological factors (age, pT status, pN status) and for the two miRNAs miR-199a-3p, miR-214-3p were used to build up two models, a full model with all five variables and a reduced model after a backward elimination approach (Table 2). This threshold of \( P \)-value <0.10 was selected to avoid a possible false negative decision (Type II error) for a potential relevant parameter using a low alpha value in this first step of model building based on univariable Cox regression analysis. Both miRNAs also remained as statistically independent variables in the model after backward elimination. In addition, the training and test set gave closely corresponding results, indicating that an overfitting bias of results can be largely excluded. It is of a special interest that the above mentioned miR-130b-3p as the best discriminator between malignant and nonmalignant bladder tissue failed to provide any prognostic information (Table 2).

To confirm the prognostic impact of miR-199-3p and miR-214-3p, \( C \)-statistics were performed in a model with the three above-mentioned relevant clinicopathological variables alone (Model CR-1) in comparison to a model (Model CR-2) that also included the relevant miR-199a-3p.

Table 2. Cox proportional hazard regression analyses of clinicopathological factors and miRNAs for predicting overall survival in MIBC patients after radical cystectomy in training and test set.

| Variable        | Training set (\( n = 100 \)) | Test set (\( n = 56 \)) |
|-----------------|-------------------------------|-------------------------|
|                 | HR (95% CI)                   | \( P \)-value           | HR (95% CI)                   | \( P \)-value           |
| Univariable analysis |
| Age (<69/≥69 years) | 1.56 (0.94–2.53)              | 0.087                   | 1.98 (0.99–3.97)              | 0.055                   |
| Sex (female/male)  | 1.61 (0.73–3.53)              | 0.283                   | 1.20 (0.45–3.62)              | 0.659                   |
| pT status (pT2,3,4) | 1.46 (1.04–2.04)              | 0.029                   | 1.64 (0.83–3.44)              | 0.098                   |
| Grade (G2/G3-4)   | 0.96 (0.46–2.01)              | 0.904                   | 1.50 (0.29–23.9)              | 0.529                   |
| pN status (N0,Nx/N1) | 2.01 (1.23–3.28)              | 0.005                   | 1.96 (0.94–4.65)              | 0.053                   |
| Adjuvant therapy  | 1.38 (0.85–2.22)              | 0.193                   | 0.89 (0.44–1.79)              | 0.741                   |
| miR-100-5p       | 0.99 (0.87–1.23)              | 0.853                   | 0.93 (0.60–1.12)              | 0.680                   |
| miR-130b-3p      | 0.99 (0.77–1.27)              | 0.181                   | 1.02 (0.53–1.85)              | 0.643                   |
| miR-141-3p       | 0.93 (0.70–1.25)              | 0.633                   | 0.76 (0.39–1.24)              | 0.306                   |
| miR-199a-3p      | 0.53 (0.30–0.91)              | 0.023                   | 0.49 (0.18–0.96)              | 0.042                   |
| miR-205-5p       | 1.02 (0.92–1.14)              | 0.720                   | 1.16 (0.81–1.95)              | 0.302                   |
| miR-214-3p       | 1.80 (1.12–2.89)              | 0.015                   | 2.96 (1.29–6.75)              | 0.005                   |
| Multivariable analysis, full model |
| Age             | 1.49 (0.88–2.51)              | 0.137                   | 1.65 (0.77–3.53)              | 0.255                   |
| pT status       | 1.45 (1.01–2.07)              | 0.045                   | 1.85 (0.99–3.43)              | 0.053                   |
| pN status       | 1.62 (0.97–2.71)              | 0.064                   | 2.26 (1.01–5.04)              | 0.046                   |
| miR-199a-3p     | 0.57 (0.32–1.02)              | 0.058                   | 0.32 (0.11–0.94)              | 0.039                   |
| miR-214-3p      | 1.79 (1.12–2.85)              | 0.015                   | 3.30 (1.11–9.77)              | 0.031                   |
| Multivariable analysis, backward elimination |
| pT status       | 1.42 (1.01–1.98)              | 0.042                   | 1.81 (0.96–3.39)              | 0.065                   |
| pN status       | 1.67 (1.01–2.77)              | 0.052                   | 2.32 (1.06–5.09)              | 0.035                   |
| miR-199a-3p     | 0.53 (0.27–0.89)              | 0.026                   | 0.35 (0.13–0.91)              | 0.032                   |
| miR-214-3p      | 1.88 (1.21–3.51)              | 0.005                   | 3.29 (1.24–8.74)              | 0.017                   |

CI, confidence interval; G, histopathological grading; HR, hazard ratio; MIBC, muscle-invasive bladder cancer; miR, microRNA; pT, pathological tumor classification; pN, lymph nodal status.

1The training set included 100 and the test set 56 of MIBC patients randomly selected from the cohort characterized in Table 1.

2Calculations were performed by bootstrapping (2000 resamples) for clinical variables using categorized data as indicated in brackets and for miRNAs using normalized continuous expression values.

3The multivariable analysis included all variables with \( P \)-values <0.10 obtained in the univariable analysis to avoid a type II error in the first step of model building.

4The backward multivariable analysis (\( P = 0.05 \) for entry; \( P = 0.10 \) for removal) was based on the five variables used in the full multivariable analysis. The 95% CI of the hazard ratios and the \( P \)-values of the final model were obtained after bootstrapping (2000 resamples).
and miR-214-3p (Table 2). Based on the median survival time of about 3 years in the entire cohort (Table 1), the AUCs in cumulative case/dynamic and incident case/dynamic control ROC analyses were calculated (Fig. 2A and C). Both approaches resulted in statistically significant higher AUC values in the miRNA-enriched model in comparison to the model with clinicopathological variables alone (AUC of 0.735 vs. 0.645 and 0.709 vs. 0.622; Fig. 2A and C). The predictive benefit of the two miRNAs is also shown in the decision curve analyses (Fig. 2B and D). The curves of the model with miRNAs (Model CR-2) are always above the curves of the model with only the clinicopathological variables (Model CR-1).

**Discussion**

In this retrospective study exclusively focused on the outcome OS, we continued to translate our conclusions drawn from previous miRNA studies in fresh-frozen tissue samples into clinical practice using archived tissue FFPE samples from three centers [18, 21]. This study design follows the concept of a discovery-driven global strategy for

---

**Figure 2.** Improved survival predictive accuracy by including miR-199a-3p and miR-214-3p (Model CR-2) in a model with only clinicopathological variables age, pT status, and lymph node metastasis (Model CR-1). Areas under the time-dependent ROC curve (AUC) of the two models were calculated based on (A) a cumulative case (n = 78)/dynamic control (n = 41) approach at 36 months after surgery as well as on (C) an incident case (n = 99)/dynamic control (n = 57) approach [24]. AUCs of Model CR-2 showed statistically significant higher values in both approaches in comparison with Model CR-1 [Model CR-2 vs. Model CR-1 in (A) with 0.735 (0.624–0.827) vs. 0.645 (0.545–0.746), P = 0.031 and in (C) with 0.709 (0.637–0.796) vs. 0.622 (0.534–0.716), P = 0.011]. Curves in the decision curve analysis confirmed (B and D) the benefit of including the two miRNAs in the model based only on clinicopathological variables.
Survival Prediction of Bladder Cancer by microRNAs

T. H. Ecke et al.

The study results suggest that miR-199a-3p and miR-214-3p would classify our present study into the third phase by clinical usability [27]. According to this scheme, we would classify our present study into the third phase. The study results suggest that miR-199a-3p and miR-214-3p are helpful biomarkers to enhance the OS prediction in comparison with clinicopathological data alone in MIBC patients after RC and improve therefore further decision-making both for clinicians and patients.

Numerous miRNA studies in bladder cancer identified histological grade and pT classification dependent miR expressions and proved typical miRNA alterations related to the two divergent pathways found in the development of NMIBC and MIBC (reviewed by Guancial et al. [28]). As already noted in the introduction, only a few studies used miRNAs as prognostic and predictive biomarkers in bladder cancer patients but mostly in NMIBC patients [9–17]. Different miRNAs were suggested as prognostic biomarkers for MIBC patients as combinations of four (let-7c, miR-125b-1, miR193a, miR-99a) [17], three (miR-9, miR-183, miR-200b) [13], and two miRNAs (miR-143, miR-145) [15], but also as predictive markers for the response to cisplatin-based adjuvant chemotherapy [11]. However, none of these studies performed multivariable analyses or verified the examined miRNAs as independent markers.

In a recent study, Schubert et al. summarized the inconsistent data situation of miRNAs as prognostic markers in urological tumors [29]. Different strategies to identify suitable prognostic miRNAs, the use of various analytical techniques, the insufficient sample size of study groups with lack of internal/external validations and multivariable analyses are reasons for the partially contradictory results if miRNAs have been applied as prognostic biomarkers in bladder cancer [29]. Furthermore, beside the application of different clinical endpoints, studies have been also performed in cohorts including both MIBC and NMIBC patients without separate data evaluation or missing multivariable analysis [16, 30]. Distinct molecular alterations including different miRNA profiles in noninvasive and invasive tumors may facilitate discrimination between both bladder cancer entities [9, 31, 32], but this aspect needs a special attention for the prognostic assessment of miRNAs. The different miRNA expression, partly characterized by an opposite expression between nonmalignant, NMIBC, and MIBC tissue as for example in the case of miR-141 and miR-205 [18], could hamper a reliable prediction of the clinical outcome in multivariable Cox regression analyses in connection with clinicopathological factors. This possible bias was impressively illustrated by the example of miR-214 [33]. This miRNA was shown to be an independent factor of recurrence-free survival and OS in the MIBC cohort but failed if MIBC and NMIBC patients were analyzed as a combined group. To avoid all these possible errors, we studied only one bladder cancer entity, MIBC patients. We used FFPE samples from the practical point of view with a sufficient sample size and number of events for carrying out multivariable analyses, and performed a twofold internal validation approach by using a training and test set with bootstrapping calculation of continuous instead of categorized data according to the REMARK guidelines to achieve a high level of statistical power.

The up- and down-regulated expression of the six selected miRNAs primarily found in fresh-frozen MIBC versus nonmalignant tissue in our previous study [18] was confirmed in the FFPE samples in this study reaching at least by a median twofold change, except for miR-205-5p (Fig. 1). Moreover, three points should be emphasized in relation to assessment of outcome as the main aim of this study: the missing or only weak correlations of miRNAs with the clinicopathological risk factors, the different correlation coefficients among the miRNAs described in the Results, and the failure of miR-130-3p as the best miRNA discriminator between nonmalignant and malignant tissue to provide any prognostic information. The striking hallmark of the uncorrelated differential expression of miRNAs to known disease variables characterizes the miRNAs here examined as potential orthogonal biomarkers [34]. This particular feature of orthogonal markers appears as a precondition that such markers can influence the clinical endpoint of OS independent of the conventional clinicopathological variables and must therefore be included as necessary independent variables in a corresponding prognostic model. The evidence of such an independent variable in multivariable analysis additionally promotes the discovery of novel downstream targets and their associated pathways [34]. Our study proved that both miR-199a-3p and miR-214-3p remained such independent variables in multivariable Cox regression models (Table 2). The predictive accuracy of OS probability based only on the conventional clinicopathological variables could be improved by these two miRNAs. This clinical benefit was verified by the decision curve analysis and also by the time-dependent ROC analysis if the two miRNAs were included into the model with only conventional clinicopathological variables (Fig. 2). The combined use of the clinicopathological variables and the expression data of the two microRNAs measured in tissue...
samples from cystectomy specimens offers the opportunity
to develop a risk scoring system in prospective studies as
result of the clinical usability of this approach. This
would correspond to the final development phase of bio-
marker assay for clinical practice as suggested above [27].
In contrast to miR-199a-3p and miR-214-3p, miR-130b-3p
as the best discriminator between nonmalignant and cancer
tissue did not offer any prognostic information. The prog-
nostic information could also not be confirmed for miR-
tissue did not offer any prognostic information. The prog-
nostic information could also not be confirmed for miR-

In contrast to miR-199a-3p and miR-214-3p, miR-130b-3p
would correspond to the final development phase of bio-
marker assay for clinical practice as suggested above [27].

As result of the clinical usability of this approach. This
would correspond to the final development phase of bio-
marker assay for clinical practice as suggested above [27].

The role of miR-199a-3p and miR-214-3p with special
focus on bladder cancerogenesis and its neoplastic pro-
gression. miR-199a derives from two loci of the human
genome, mir-199a-1 of chromosome 19 (cytogenetic loca-
tion 19p13.2) and mir-199a-2 of chromosome 1 (cytoge-
netic location 1q24.3). These two loci encode mir-199a,
which generate the two mature miRNAs, miR-199a-3p
and miR-199a-5p. Depending on the underlying malign-
nancy, miR-199a-3p acts both as oncogene being up-
regulated like in gastric and colorectal cancer [35, 36]
and as tumor suppressor being down-regulated like in
renal cell cancer, hepatocellular carcinoma, prostate cancer
or bladder cancer [37–39]. The down-regulated expression
of miR-199a-3p in bladder cancer was also described as
tumor suppressive miRNA by other groups but not further
evaluated as prognostic marker [31, 32, 40, 41]. After we
had finished our study, Sakaguchi et al. [42] recently
showed that the expression of all four mir-199 family
members consisting of miR-199b-3p, and miR-199b-5p
in addition to the two above-mentioned mir-199a forms,
was down-regulated in bladder cancer. The decreased
expression of the miRNAs was found to be associated
with a poor OS of patients shown in Kaplan–Meier analy-
sis but it was not additionally assessed in multivariable Cox
regression analyses [42]. It was shown that these miRNAs
act as tumor suppressors by targeting ITGA3 (integrin
subunit α3). However, the increased ITGA3 mRNA expres-
sion was not significantly associated with the survival of
patients [42]. This phenomenon of missing link between a
differentially expressed miRNA and its verified target
gene with regard to a clinical endpoint is understandable,
bearing in mind that miR-199a targets numerous genes
that could affect the prognosis of patients. For example,
miR-199a-3p was validated as tumor suppressive miRNA
in other cancers like prostate cancer targeting AURKA
(aurora kinase A) [39], in renal cell cancer GSK3B (gly-
cogen synthase kinase 3 beta) [37], and in hepatocellular
carcinoma HGF (hepatect growth factor), MMP2 (matrix
metalloproteinase 2), VEGFA (vascular endothelial growth
factor A), and its corresponding receptors [43]. So far,
these targets have not been examined in bladder cancer
as potential points of action for miR-199a-3p. However,
all these genes play a significant role in signaling pathways
of angiogenesis, invasion, and metastasis in cancer as
shown in the software DIANA-miRPath (http://diana.imis.
athena-innovation.gr/DianaTools) [44]. Moreover, it is of
particular interest that the second relevant prognostic
mature miRNA in our study, miR-214-3p, derives from the
stem loop sequence of mir-214 that clusters with mir-
199a-2 of the same chromosome locus 1q24.3. Similar to
miR-199a-3p, miR-214-3p acts, depending on the cancer
types, oncogenic in osteosarcoma and oral cancer [45,
46] but tumor suppressive in breast, cervical, esophageal,
and bladder cancers [33, 47–51]. Based on our previous
expression study [18], Wang et al. [33] characterized the
down-regulated miR-214-3p as a suppressive miRNA. This
miRNA functions as an independent factor of OS in MIBC
patients, targeting the oncogene PDRG1 (p53 and DNA
damage regulated 1). SLC34A2 (solute carrier family 34
member 2) and several genes of the epithelial-mesenchymal
transition and the NGAL/MMP-9 (lipocalin 2/metallopro-
tidase 9) pathways were also verified or predicted to be
negatively regulated by miR-214-3p in the process of
bladder cancer development [50, 51].

The possible biological significance of the co-expression
of the two down-regulated miR-199a and miR-214 was
shown in testicular germ cell tumor [52]. A self-regulatory
network of the two microRNAs together with PSMD10
(proteasome 26S subunit, non-ATPase 10), TP53 (tumor
protein p53), and DNTM1 (DNA methyltransferase 1) was
identified whose dysfunction results in tumor progression.
As mentioned above, these two microRNAs also act as
regulators of numerous target genes that have been already
validated so far in urinary bladder and other solid cancers
(target characteristics are given in Table S4). Moreover,
based on our data of prognostic relevance of miR-199a-3p
and miR-214-3p, the view is supported that such a regula-
tory network or at least a cooperative action of the micro-
RNAs as shown in other cancer examples [53] could also
exist in bladder cancer. A detailed investigation of the
functional role of the two miRNAs would be worthwhile
but was beyond the scope of this study. On the other
hand, we share the view of Burke [54] that the functional
role of biomarker might be, in a certain sense, useful to
explain the biological rationale of biomarker but is not
decisive for its clinical usability. Under this translational
aspect, the primary focus in the search for a truly reliable
biomarker has to be directed more toward the clinical
benefit of the potential new biomarker in comparison to
the so far used procedures in clinical practice.
Limitations of the study are, despite our precautions for bias-free analyses using the internal validation by a training and test sets with bootstrapping, its retrospective nature on overall and not cancer-specific survival, the lack of external validation, and the biomarker-focused aspect without elucidating the molecular mechanisms as argued above.

Conclusions
In summary, our study identified miR-199a-3p and miR-214-3p as independent prognostic biomarkers for the prediction of OS in MIBC patients after RC. Their inclusion in prognostic models based on relevant clinicopathological risk factors improved the predictive accuracy. Such enhanced information of combined clinicopathological and molecular data could support clinicians in their decision-making in the treatment management and follow-up scheduling after RC. It could also help patients who wish to be kept fully informed about their further life expectancy to plan and manage their remaining lifetime. Thus, further efforts through complimentary multicenter prospective randomized studies would be worth to translate these biomarkers into clinical practice as suggested.

Acknowledgements
The support by the Berliner Krebsgesellschaft (Grant no. JUFF201403), the SONNENFELD-STIFTUNG Berlin (Grant no. 25.3.2015/GB/co), and the Foundation of Urologic Research (Grant no. SKJ_02_2014) is gratefully acknowledged. The grant sponsors had no role in the interpretation of the data; the writing of the manuscript; and the decision to submit the manuscript.

Conflict of Interest
None declared.

References
1. Ferlay, J., E. Steliarova-Foucher, J. Lortet-Tieulent, S. Rosso, J. W. Coebergh, H. Comber, et al. 2013. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur. J. Cancer 49:1374–1403.
2. Witjes, J. A., E. Comperat, N. C. Cowan, S. M. De, G. Gakis, T. Lebret, et al. 2014. EAU guidelines on muscle-invasive and metastatic bladder cancer: summary of the 2013 guidelines. Eur. Urol. 65:778–792.
3. Witjes, J. A., T. Lebret, E. M. Comperat, N. C. Cowan, S. M. De, H. M. Bruins, et al. 2017. Updated 2016 EAU Guidelines on muscle-invasive and metastatic bladder cancer. Eur. Urol. 71:462–475.
4. Kluth, L. A., P. C. Black, B. H. Bochner, J. Catto, S. P. Lerner, A. Stenzl, et al. 2015. Prognostic and prediction tools in bladder cancer: a comprehensive review of the literature. Eur. Urol. 68:238–253.
5. Hoffmann, A. C., P. Wild, C. Leicht, S. Bertz, K. D. Danenberg, P. V. Danenberg, et al. 2010. MDR1 and ERCC1 expression predict outcome of patients with locally advanced bladder cancer receiving adjuvant chemotherapy. Neoplasia 12:628–636.
6. Cheng, L., D. D. Davison, J. Adams, A. Lopez-Beltran, L. Wang, R. Montironi, et al. 2014. Biomarkers in bladder cancer: translational and clinical implications. Crit. Rev. Oncol. Hematol. 89:73–111.
7. Kim, W. J., S. K. Kim, P. Jeong, S. J. Yun, I. C. Cho, I. Y. Kim, et al. 2011. A four-gene signature predicts disease progression in muscle invasive bladder cancer. Mol. Med. 17:478–485.
8. Mitra, A. P., L. L. Lam, M. Ghadessi, N. Erho, I. A. Vergara, M. Alshalafla, et al. 2014. Discovery and validation of novel expression signature for postcystectomy recurrence in high-risk bladder cancer. J. Natl Cancer Inst. 106:du1290.
9. Catto, J. W., S. Miah, H. C. Owen, H. Bryant, K. Myers, E. Dudziec, et al. 2009. Distinct microRNA alterations characterize high- and low-grade bladder cancer. Cancer Res. 69:8472–8481.
10. Neely, L. A., K. M. Rieger-Christ, B. S. Neto, A. Eroshkin, J. Garver, S. Patel, et al. 2010. A microRNA expression ratio defining the invasive phenotype in bladder tumors. Urol. Oncol. 28:39–48.
11. Nordentoft, L., K. Birkenkamp-Demtroder, M. Agerbaek, D. Theodorescu, M. S. Ostenfeld, A. Hartmann, et al. 2012. miRNAs associated with chemo-sensitivity in cell lines and in advanced bladder cancer. BMC Med. Genomics 5:40.
12. Puerta-Gil, P., R. Garcia-Baquero, A. Y. Jia, S. Ocana, M. Alvarez-Mugica, J. L. Alvarez-Ossorio, et al. 2012. miR-143, miR-222, and miR-452 are useful as tumor stratification and noninvasive diagnostic biomarkers for bladder cancer. Am. J. Pathol. 180:1808–1815.
13. Pignot, G., G. Cizeron-Clairac, S. Vacher, A. Susini, S. Tozlu, A. Vieillefond, et al. 2013. microRNA expression profile in a large series of bladder tumors: identification of a 3-miRNA signature associated with aggressiveness of muscle-invasive bladder cancer. Crit. Rev. Oncol. Hematol. 89:73–111.
14. Andrew, A. S., C. J. Marsit, A. R. Schned, J. D. Seignie, K. T. Kelsey, J. H. Moore, et al. 2015. Expression of tumor suppressive microRNA-34a is associated with a reduced risk of bladder cancer recurrence. Int. J. Cancer 137:1158–1166.
15. Avgeris, M., K. Mavridis, T. Tokas, K. Stravodimos, E. G. Fragoulis, and A. Scorilas. 2015. Uncovering the clinical
utility of miR-143, miR-145 and miR-224 for predicting the survival of bladder cancer patients following treatment. Carcinogenesis 36:528–537.

16. Wang, X. L., H. Y. Xie, C. D. Zhu, X. F. Zhu, G. X. Cao, X. H. Chen, et al. 2015. Increased miR-141 expression is associated with diagnosis and favorable prognosis of patients with bladder cancer. Tumour Biol. 36:877–883.

17. Xu, Z., Y. Q. Yu, Y. Z. Ge, J. G. Zhu, M. Zhu, Y. C. Zhao, et al. 2015. MicroRNA expression profiles in muscle-invasive bladder cancer: identification of a four-microRNA signature associated with patient survival. Tumour Biol. 36:8159–8166.

18. Ratert, N., H.-A. Meyer, M. Jung, P. Lioudmer, H.-J. Mollenkopf, I. Wagner, et al. 2013. MicroRNA profiling identifies candidate miRNAs for bladder cancer diagnosis and clinical outcome. J. Mol. Diagn. 15:695–705.

19. Altman, D. G., L. M. McShane, W. Sauerbrei, and S. E. Taube. 2012. Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK): explanation and elaboration. PLoS Med. 9:e1001216.

20. Bossuyt, P. M., J. B. Reitsma, D. E. Bruns, C. A. Gatsonis, P. P. Glasziou, L. Irwig, et al. 2015. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. Clin. Chem. 61:1446–1452.

21. Ratert, N., H.-A. Meyer, M. Jung, H.-J. Mollenkopf, I. Wagner, K. Miller, et al. 2012. Reference miRNAs for miRNAome analysis of urothelial carcinomas. PLoS ONE 7:e39309.

22. Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, et al. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55:611–622.

23. Vickers, A. J., and E. B. Elkin. 2006. Decision curve analysis: a novel method for evaluating prediction models. Med. Decis. Making 26:565–574.

24. Heagerty, P. J., and Y. Zheng. 2005. Survival model predictive accuracy and ROC curves. Biometrics 61:92–105.

25. Marincola, F. M. 2003. Translational medicine: a two-way road. J. Transl. Med. 1:1.

26. Littman, B. H., M. L. Di, M. Plebani, and F. M. Marincola. 2007. What’s next in translational medicine? Clin. Sci. 112:217–227.

27. Fendler, A., C. Stephan, G. M. Yousef, G. Kristiansen, and K. Jung. 2016. The translational potential of microRNAs as biofluid markers of urologic tumors. Nat. Rev. Urol. 13:734–752.

28. Guancial, E. A., J. Bellmunt, S. Yeh, J. E. Rosenberg, and D. M. Berman. 2014. The evolving understanding of microRNA in bladder cancer. Urol. Oncol. 32:41.

29. Schubert, M., K. Junker, and J. Heinzelmann. 2016. Prognostic and predictive miRNA biomarkers in bladder, kidney and prostate cancer: where do we stand in biomarker development? J. Cancer Res. Clin. Oncol. 142:1673–1695.

30. Wang, S., Q. Li, K. Wang, Y. Dai, J. Yang, S. Xue, et al. 2013. Decreased expression of microRNA-31 associates with aggressive tumor progression and poor prognosis in patients with bladder cancer. Clin. Transl. Oncol. 15:849–854.

31. Dyryskjot, L., M. S. Ostenfeld, J. B. Bramsen, A. N. Silahtaroglu, P. Lamy, R. Ramanathan, et al. 2009. Genomic profiling of microRNAs in bladder cancer: miR-129 is associated with poor outcome and promotes cell death in vitro. Cancer Res. 69:4851–4860.

32. Rosenberg, E., J. Daniel, Y. Spector, A. Faerman, E. Meiri, R. Aharonov, et al. 2013. Predicting progression of bladder urothelial carcinoma using microRNA expression. BJU Int. 112:1027–1034.

33. Wang, J., X. Zhang, L. Wang, Y. Yang, Z. Dong, H. Wang, et al. 2015. MicroRNA-214 suppresses oncogenesis and exerts impact on prognosis by targeting PDRG1 in bladder cancer. PLoS ONE 10:e0118086.

34. Gerszten, R. E., and T. J. Wang. 2008. The search for new cardiovascular biomarkers. Nature 451:949–952.

35. Wang, Z., X. Ma, Q. Cai, X. Wang, and B. Yu. 2014. Cai Q, liu B, Zhu Z, Li C. MiR-199a-3p promotes gastric cancer progression by targeting ZHX1. FEBS Lett. 588:4504–4512.

36. Wen, D., S. He, B. Xie, G. Xu, W. Gu, C. Shen, et al. 2013. Aberrant expression of miR-199a-3p and its clinical significance in colorectal cancers. Med. Oncol. 30:378.

37. Tsukigi, M., V. Bilim, K. Yuuki, A. Ugolkov, S. Naito, A. Nagaoka, et al. 2012. Re-expression of miR-199a suppresses renal cancer cell proliferation and survival by targeting GSK-3beta. Cancer Lett. 315:189–197.

38. Amr, K. S., W. M. Ezzat, Y. A. Elhosary, A. E. Hegazy, H. H. Fahim, and R. R. Kamel. 2016. The potential role of miRNAs 21 and 199-a in early diagnosis of hepatocellular carcinoma. Gene 575:66–70.

39. Qu, Y., X. Huang, Z. Li, J. Liu, J. Wu, D. Chen, et al. 2014. mir-199a-3p inhibits aurora kinase A and attenuates prostate cancer growth: new avenue for prostate cancer treatment. Am. J. Pathol. 184:1541–1549.

40. Ichimi, T., H. Enokida, Y. Okuno, R. Kunimoto, T. Chiyomaru, K. Kawamoto, et al. 2009. Identification of novel microRNA targets based on microRNA signatures in bladder cancer. Int. J. Cancer 125:345–352.

41. Li, X., J. Chen, X. Hu, Y. Huang, Z. Li, L. Zhou, et al. 2011. Comparative mRNA and microRNA expression profiling of three genitourinary cancers reveals common hallmarks and cancer-specific molecular events. PLoS ONE 6:e22570.
42. Sakaguchi, T., H. Yoshino, M. Yonemori, K. Miyamoto, S. Sugita, R. Matsushita, et al. 2017. Regulation of ITGA3 by the dual-stranded microRNA-199 family as a potential prognostic marker in bladder cancer. Br. J. Cancer 116:1077–1087.

43. Ghosh, A., D. Dasgupta, A. Ghosh, S. Roychoudhury, D. Kumar, M. Gorain, et al. 2017. MiRNA199a-3p suppresses tumor growth, migration, invasion and angiogenesis in hepatocellular carcinoma by targeting VEGFA, VEGFR1, VEGFR2, HGF and MMP2. Cell Death Dis. 8:e2706.

44. Vlachos, I. S., N. Kostoulas, T. Vergoulis, G. Georgakilas, M. Reczko, M. Maragkakis, et al. 2012. DIANA miRPath v. 2.0: investigating the combinatorial effect of microRNAs in pathways. Nucleic Acids Res. 40:W498–W504.

45. Zhu, X. B., Z. C. Zhang, G. S. Han, J. Z. Han, and D. P. Qiu. 2017. Overexpression of miR214 promotes the progression of human osteosarcoma by regulating the Wnt/betacatenin signaling pathway. Mol. Med. Rep. 15:1884–1892.

46. Li, T. K., K. Yin, Z. Chen, Y. Bao, and S. X. Zhang. 2017. MiR-214 regulates oral cancer KB cell apoptosis through targeting RASSF5. Genet. Mol. Res. 16:gmr16019327.

47. Zhang, J., B. Su, C. Gong, Q. Xi, and T. Chao. 2016. miR-214 promotes apoptosis and sensitizes breast cancer cells to doxorubicin by targeting the RFWD2-p53 cascade. Biochem. Biophys. Res. Commun. 478:337–342.

48. Peng, R., J. Men, R. Ma, Q. Wang, Y. Wang, Y. Sun, et al. 2017. miR-214 down-regulates ARL2 and suppresses growth and invasion of cervical cancer cells. Biochem. Biophys. Res. Commun. 484:623–630.

49. Phatak, P., K. A. Byrnes, D. Mansour, L. Liu, S. Cao, R. Li, et al. 2016. Overexpression of miR-214-3p in esophageal squamous cancer cells enhances sensitivity to cisplatin by targeting survivin directly and indirectly through CUG-BP1. Oncogene 35:2087–2097.

50. Falzone, L., S. Candido, R. Salemi, M. S. Basile, A. Scalisi, J. A. McCubrey, et al. 2016. Computational identification of microRNAs associated to both epithelial to mesenchymal transition and NGAL/MMP-9 pathways in bladder cancer. Oncotarget 7:72758–72766.

51. Ye, W., C. Chen, Y. Gao, Z. S. Zheng, Y. Xu, M. Yun, et al. 2017. Overexpression of SLC34A2 is an independent prognostic indicator in bladder cancer and its depletion suppresses tumor growth via decreasing c-Myc expression and transcriptional activity. Cell Death Dis. 8:e2581.

52. Chen, B. F., Y. K. Suen, S. Gu, L. Li, and W. Y. Chan. 2014. A miR-199a/miR-214 self-regulatory network via PSMD10, TP53 and DNMT1 in testicular germ cell tumor. Sci. Rep. 4:6413.

53. Liep, J., E. Kilic, H. A. Meyer, J. Busch, K. Jung, and A. Rabien. 2016. Cooperative effect of miR-141-3p and miR-145-5p in the regulation of targets in clear cell renal cell carcinoma. PLoS ONE 11:e0157801.

54. Burke, H. B. 2016. Predicting clinical outcomes using molecular biomarkers. Biomark. Cancer 8:89–99.

**Supporting Information**

Additional supporting information may be found in the online version of this article:

- **Table S1.** Details of the TaqMan microRNA assays.
- **Figure S1.** Discriminative capacity of miRNA models.
- **Table S2.** Receiver-operating characteristics analyses of miRNAs and their combinations with comments to Figure S1 and data in Table S2.
- **Table S3.** Spearman correlation coefficients of miRNA-pairs with comments to the data.
- **Figure S2.** Kaplan–Meier analyses of overall survivals of muscle-invasive bladder cancer patients after radical cystectomy in association with clinicopathological variables.
- **Table S4.** Target genes of miR-199a-3p and miR-214-3p.