CDKN2A as transcriptomic marker for muscle-invasive bladder cancer risk stratification and therapy decision-making

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Deletions of the cell cycle control gene CDKN2A are described as progression markers of non-muscle invasive bladder cancer and to be associated with fibroblast growth factor 3 (FGFR3) mutations. The prognostic role of CDKN2A RNA expression in muscle invasive bladder cancer (MIBC) is under discussion. In 80 MIBC patients (m/f 60/20) who underwent radical cystectomy the expression of CDKN2A and FGFR3 was examined with qRT-PCR (test cohort). The MDA cohort (n = 57) and the TCGA cohort (n = 365) served for validation. The expression of drug target genes and TCGA molecular subtypes was correlated with CDKN2A expression. In the test cohort CDKN2A high patients (n = 8; 10.0%) had a significantly shorter recurrence-free (p = 0.018) and disease-specific (p = 0.006) survival compared to the rest of the cohort. A similar stratification was seen in the validation cohorts (CDKN2A high: n = 7, 12.3%, p = 0.001; n = 46, 12.6%, p = 0.011). In the TCGA cohort these patients had a comparably low expression of drug target genes. The expression of CDKN2A significantly differed among TCGA molecular subtypes. 71.7% of CDKN2A high were TCGA basal squamous tumours but also show divergent molecular features compared to this group. In summary CDKN2A RNA expression-based risk stratification of MIBC allows the identification of a CDKN2A high poor prognosis group with low expression of drug target genes.

For several decades radical cystectomy (RC) is the standard therapy of muscle invasive bladder cancer (MIBC). Yet, due to a high recurrence rate, 5-year overall survival (OS) of patients with locally advanced tumours is only around 50%. In the hospital routine decisions on adjuvant, neoadjuvant and palliative medication still mainly rely on clinical parameters. Though deemed crucial in terms of risk stratification and identification of patients in need for a more aggressive treatment, molecular profiling for individual therapy decision-making is still in its infancy in MIBC. Furthermore, expression data can give valuable information about drug target gene expression.

In the light of bladder cancer initiation several frequent genetic aberrations have been identified. Papillary/non muscle-invasive and non-papillary/muscle-invasive bladder cancer are typically seen as two different molecular entities. In both groups alterations of “forerunner genes” are seen as an initial event. Whilst in papillary tumours, genetic alterations are mainly restricted to these genes, high risk NMIBC and MIBC often show alterations of major tumour suppressor genes as RB1 or TP53.

Loss of heterozygosity (LOH) in the 9p region is one of this typical early events in the formation of bladder cancer and frequently occurs in non-invasive precursor lesions like hyperplasia, dysplasia or carcinoma in situ. One of the genes found in this region is CDKN2A, which codes for the cell cycle control protein p16. LOH of

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CDKN2A and decreased expression of the p16 protein are mainly described as a predictor of progression in non muscle-invasive bladder cancer (NMIBC)\(^\text{14}\). Homozygous deletion of CDKN2A, is also associated with muscle invasion in FGFR3-mutated (fibroblast growth factor receptor 3) tumours\(^\text{15}\).

On the protein level a meta-analysis\(^\text{16}\), including data from 17 immunohistochemistry studies with 1032 subjects, investigated the p16 expression in various disease stages and found a significant association between a low expression of p16 and recurrence-free survival in patients with all stages of bladder cancer. When stratifying for T stages this correlation was markedly stronger for NMIBC, but was not found for MIBC (\(\geq \text{T2}\)). The same was found for progression-free survival (PFS). The authors concluded that the p16 expression is affected by clinicopathologic stage and its relevance is mainly to be seen in NMIBC.

Another study found altered p16 protein expression, defined as either no expression of p16 or a very strong expression (\(\geq 2\) times the expression of p16) in various disease stages and found a significant association between a low expression of p16 and recurrence-free survival in patients with all stages of bladder cancer. When stratifying for T stages this correlation was markedly stronger for NMIBC, but was not found for MIBC (\(\geq \text{T2}\)). The same was found for progression-free survival (PFS). The authors concluded that the p16 expression is affected by clinicopathologic stage and its relevance is mainly to be seen in NMIBC.

We therefore aimed to stratify patients with MIBC according to their CDKN2A expression. Since immunohistochemistry is limited in case of quantification and sample comparison, RNA-based methods like qRT-PCR or next generation sequencing are robust alternatives for quantification and stratification of gene expression. The value of CDKN2A mRNA expression has not been systematically investigated in MIBC, yet, but qRT-PCR has already proved to be a valuable tool to determine CDKN2A copy number status\(^\text{18}\). The CDKN2A RNA-expression-based risk stratification was validated in the MDA and the TCGA cohort. Furthermore we reanalysed TCGA data to reveal correlations of CDKN2A with drug target gene expression and molecular subtypes.

**Results**

**CDKN2A RNA expression allows risk stratification of MIBC patients.** When stratifying for disease-specific death using the partition test, the test cohort of 80 patients with MIBC could be divided into two groups with different CDKN2A expression (CDKN2A\(^\text{high}\) with \(n = 8\), 10.0%; CDKN2A\(^\text{low}\) with \(n = 72\), 90.0%). Clinicopathologic data did not differ significantly between these groups (Table 1).

| parameter | total (n = 80) | CDKN2A | p-value (Chi²) |
|-----------|---------------|--------|----------------|
| Male      | 60 (75.0%)    | 55 (76.4%) | p = 0.389      |
| Female    | 20 (25.0%)    | 17 (23.6%)  |                 |

Kaplan-Meier analysis for recurrence-free survival (RFS, Fig. 1a) and disease-specific survival (DSS, Fig. 1b) showed significant differences (\(p = 0.018\) and \(p = 0.006\)) between these groups, with CDKN2A\(^\text{high}\) having a worse prognosis (median RFS 16.3 months and median DSS 11.2 months) compared to patients with CDKN2A\(^\text{low}\) tumours (median RFS 74.2 months and median DSS 131.7 months).

By using the partition test in the MDA cohort of 57 bladder cancer patients a similar cut-off for CDKN2A expression could be defined. As in the test cohort, those patients with the highest CDKN2A expression (\(n = 7\); 12.3%) had a worse prognosis (\(p = 0.001\); median DSS 25.3 months, for CDKN2A\(^\text{low}\) median DSS was not reached; Fig. 2a).

When applying the partition test to the TCGA cohort, again a small group of patients with the highest CDKN2A expression (\(n = 46\); 12.6%) with a poor prognosis could be identified (\(p = 0.011\); Fig. 2b). The median overall survival (OS) of CDKN2A\(^\text{high}\) was 18.0 months, compared to 38.2 months in the CDKN2A\(^\text{low}\) group.

**Table 1.** Patient characteristics of the test cohort.

| parameter | total (n = 57) | CDKN2A | p-value (Chi²) |
|-----------|---------------|--------|----------------|
| Male      | 49 (85.9%)    | 43 (86.0%) | p = 0.984      |
| Female    | 8 (14.1%)     | 7 (14.0%)   |                 |

**Table 2.** Patient characteristics of the MDA cohort.

| parameter | total (n = 57) | CDKN2A | p-value (Chi²) |
|-----------|---------------|--------|----------------|
| Male      | 26 (45.6%)    | 19 (38.0%) | p = 0.969      |
| Female    | 31 (54.4%)    | 23 (42.9%)  |                 |
Clinicopathologic data did not differ significantly between the CDKN2A expression groups in the MDA and the TCGA cohort (Table 2 and Table 3).

Further dissection of the bigger group of CDKN2A low tumours in the test cohort and the TCGA cohort did not result in similar subgroup sizes, but yet resulted in different cut-offs with significant differences in prognosis, with patients with an intermediate expression of CDKN2A having a better prognosis as those with a low expression (Supplementary Figure 1).

Expression of drug target genes in dependence on CDKN2A expression. In the TCGA cohort there was a negative correlation between CDKN2A and FGFR3 in all MIBC (\( \rho = -0.406; p < 0.001 \)). In the test cohort there was also a trend towards a negative correlation between FGFR3 and CDKN2A (\( \rho = -0.217; p = 0.053 \)). Yet, inter-group comparison did not show a significant difference in FGFR3 expression between CDKN2A low and CDKN2A high tumours (\( p = 0.0493 \); Fig. 3a). In the TCGA cohort the FGFR3 expression differed significantly between the CDKN2A expression groups (\( p < 0.001 \); Fig. 3b).

Stratification for CDKN2A expression also showed a significantly lower expression of ESR2 in CDKN2A high tumours (\( p < 0.001 \), Fig. 3c). For the other tested drug target genes (AR, ESR1, ERBB2, PDCD1, CD274 and CTLA4) no significant differences in the two CDKN2A expression groups were seen. Over all MIBC patients from the TCGA cohort AR was negatively correlated with CDKN2A expression (\( \rho = -0.183; p = 0.004 \)) and PDCD1, CD274 and CTLA4 were positively correlated with CDKN2A expression (\( p = 0.176; p < 0.001; p = 0.327; p < 0.001; p = 0.171; p < 0.001 \)). Detailed results are given in Supplementary Table 1.
**CDKN2A CNV status, downstream target expression and molecular subtypes.** In the TCGA cohort patients with CDKN2A low expression frequently had CDKN2A deletions (38.9% homozygous deletion, heterozygous deletions: 26.0%) in comparison to more balanced genotypes in the CDKN2A high group (−1: 21.7%, balanced: 34.8%, +1: 43.5%, Supplementary Figure 2a). Chi² test proved this difference to be significant (p < 0.001). Vice versa, upon stratification of CDKN2A expression for CDKN2A CNV there was also a significant difference between CNV groups (Kruskal-Wallis p < 0.001, Supplementary Figure 2b).

Supplementary Figure 3a illustrates the CDKN2A expression in the CDKN2A expression groups of the TCGA cohort. CDK4, despite being negatively regulated by CDKN2A, showed a slightly, but not significantly higher expression in CDKN2A high (p < 0.139; Supplementary Figure 3b). RB1, which is regulated by CDK4, showed a significantly lower expression in CDKN2A high (p < 0.001, Supplementary Figure 3c) and the downstream transcription factor gene E2F3 was significantly higher expressed in CDKN2A high tumours (p < 0.001, Supplementary Figure 3d). In the complete cohort a positive correlation was observed between CDKN2A and CDK4 gene expression (r = 0.222, p < 0.001) and E2F3 gene expression (r = 0.212, p < 0.001) and a negative correlation between CDKN2A and RB1 (r = −0.479, p < 0.001).

When correlated with copy number status, there was a significant difference in the distribution of updated TCGA subtypes (Chi² p = 0.016; Table 4 and Fig. 4a). Tumours with basal squamous and neuronal expression...
phenotype were overrepresented in the group of tumours with no deletion. Luminal tumours mainly had a homozygous deletion. Tumours from the basal squamous group on average showed the highest CDKN2A expression and tumours from the luminal group had a comparably low CDKN2A expression. Overall groups Kruskal-Wallis test showed a significantly different distribution, with 33 of 46 tumours (71.7%) in the CDKN2A high group being classified as basal squamous (Fig. 4b).

When looking at specific genes, typically determining RNA expression subtypes, GATA3 ($\rho = -0.162$; $p = 0.002$) and FOXA1 ($\rho = -0.299$; $p < 0.001$) showed an inverse correlation with the CDKN2A expression. Both genes also showed a significantly lower expression in CDKN2A high tumours (both $p < 0.001$; Fig. 4d,e). Furthermore KRT20 was significantly lower expressed in CDKN2A high tumours ($p = 0.028$; Fig. 4c). Detailed results are given in Supplementary Table 2.

### Discussion

Deletions of CDKN2A and the underexpression of p16, the protein coded by CDKN2A, are well-investigated molecular risk factors for tumour progression in NMIBC. Based on this, one could conclude that deletion or underexpression of CDKN2A/p16 is also an indicator of increased aggressiveness and worse prognosis in MIBC. Yet, some data point to a more complex situation in MIBC. For instance, gene expression studies have shown distinct RNA expression patterns for Ta tumours and MIBC, with T1 tumours showing either one or the other signature. To more deeply investigate the role of CDKN2A expression in tumour prognosis and its association with
drug target genes like FGFR3, we performed qRT-PCR expression profiling and reanalysis of existing CDKN2A and FGFR3 RNA expression data of MIBC after RC. Compared to immunohistochemistry studies of p16, RNA-testing with qRT-PCR has the advantages of a higher dynamic width and a higher sensitivity. Furthermore qRT-PCR testing allows an observer-independent interpretation of quantitatively results.

In the three analyzed cohorts the groups with the highest expression of CDKN2A (10.0–12.6% of the examined patients) were identified to have a worse prognosis compared to the remaining patients. This is controversial to previous assumptions derived from findings in NMIBC, where deletion of CDKN2A, typically going along with a lowered expression, is deemed as a marker for poor prognosis21,22.

However, already in 2004 it was shown that both a low and a high expression of the p16 protein can be a predictor of worse outcome after RC17. The overall prevalence of altered p16 protein expression was 54% of the analyzed tumours. The results of last-mentioned study show that both high and low expression of CDKN2A and p16, respectively, are associated with a worse outcome of MIBC patients. Though we could not find similar cut-off values in the different cohorts to distinguish between patients with low and intermediate expression of CDKN2A in the present study, patients with an intermediate expression seem to have the best prognosis, pointing to a diverse role of CDKN2A as prognosis marker in MIBC.

Functionally it is well known that impaired function or expression of p16, either due to CDKN2A deletion, mutation or hypermethylation, leads to cell cycle deregulation via overactivation of CDK4 and CDK6, which results in hyperphosphorylation of retinoblastoma protein (RB), the protein product of RB1. The subsequent liberation of E2F transcription factor family members mediates changes in gene expression, promoting the transition from G1 to S phase21. Besides this mechanism, loss of RB1 also results in tumour formation and progression21,22. The close relation between CDKN2A/p16 and RB has been repeatedly described in urinary bladder cancer21,22. Yet, this does not intuitively explain why those MIBC with the highest CDKN2A expression show a poor prognosis. Sjödahl et al. reported different two genomic circuits operative in urothelial carcinomas: one defined by high FGFR3 and CCND1 expression, low CDKN2A expression, often associated with CDKN2A loss and the other one defined by E2F amplifications and overexpression, RB1 deletions and low expression and high CDKN2A/p16 expression27. Whilst the first circuit is mainly found in tumours termed urobasal A and urobasal B, the latter circuit was mainly associated with genomically unstable tumours29,28. These tumours also on the protein level typically showed no or low expression of KRT5 and KRT14, aberrant expression of KRT20 and a low expression of EGRF, but a high expression of ERBB2. According to this immunohistochemistry-based classification, genetically unstable tumours had a worse DSS compared to uroblastic tumours, but than squamous cancer cell-like tumours in a mixed population of NMIBC and MIBC27. Recent work from our own group has also shown high CDKN2A expression to be associated with shorter progress-free survival in T1 urothelial carcinoma29. The group of CDKN2Ahigh MIBC consistently identified in a proportion between 10.0 and 12.6% in all three datasets analyzed in the present study therefore might reflect a subgroup of genomically instable tumours, which account for 21.5% of advanced bladder cancers as described by Sjödahl et al.30. The reported overexpression of CDKN2A in genomically unstable tumours could be a sign of an in vain countermeasure to reduce cell cycle activity, which is deregulated due to other molecular aberrations.

Controversial to an association with the genomically unstable subtype is the fact, that 71.7% of the tumours with CDKN2Ahigh from the TCGA cohort are termed as basal squamous according to the 2017 TCGA publication31. Yet, this group (35% of MIBC in the TCGA cohort), also comprises 41% of tumours with CDKN2A deep deletions, meaning that CDKN2Ahigh tumours, which do not show any homozygous deletion of CDKN2A, are in part not a representative, but a highly selected subgroup of basal squamous tumours. In line with this and unlike reported for basal squamous tumours in the TCGA publication, CDKN2Ahigh tumours also do not show an elevation of PDCD1, CD274 and CTLA4 expression.

With regard to drug target gene expression, CDKN2A expression showed a negative correlation with FGFR3 expression in the TCGA cohort and a trend towards a negative correlation in the test cohort. The TCGA publication from 2014 proposed a correlation between CDKN2A deletion or underexpression and activating mutations of FGFR3 or FGFR3 overexpression1. They also reported an inverse correlation between CDKN2A and FGFR3 RNA expression. According to their mutational data they proposed three subtypes of bladder cancer: (A) focally amplified, (B) papillary CDKN2A-deficient and FGFR3-mutant and (C) TP53/cell-cycle-mutant. The study of Rebourissou and colleagues confirmed a high incidence of CDKN2A deletions (hemizygous 23.7%, homozygous 17.5%) and FGFR3 mutations (62.1%) in NMIBC15. In MIBC the rates of CDKN2A deletions were even higher (hemizygous 27.9%, homozygous 22.5%). Both in NMIBC and in MIBC there was a significant coincidence of CDKN2A deletions and activating mutations of FGFR3 and NMIBC tumours with this feature had an increased progression rate.

Due to their low FGFR3 expression, patients with CDKN2Ahigh tumours presumably do not seem to be suitable candidates for a therapy targeting FGFR3, whilst patients with a high FGFR3 expression might benefit from such an approach. The tyrosine kinases inhibitor Pazopanib is already approved for the treatment of advanced or metastatic kidney cancer and certain sarcoma entities, but there is only limited data about its application in MIBC. A small phase II trial on 19 unselected patients with metastatic bladder cancer reported a median PFS of only 1.9 months31. Another phase II study of 41 unselected patients with metastatic bladder cancer after failure of chemotherapy reported an overall initial response rate of 17%, but PFS and OS were poor32. However, there were also two patients with sustained long-term response. The RNA expression of FGFR3 in these tumours is not reported in the trial. Another group reported a case of a woman with a metastatic bladder cancer carrying an activating FGFR3 mutation33. This patient showed a durable remission of more than 6 months upon treatment with Pazopanib. In vitro results also suggest a synergistic effect of Pazopanib with Docetaxel in the treatment of bladder cancer cells34, pointing to a potential role of Pazopanib in combination therapy of cases with a suitable molecular
profile. Besides Pazopanib, several other substances targeting FGFRs are currently under investigation and AZ12908010, AZD4547, PD173074, TKI-258/Dovitinib, SU5402 and BGI-398 showed promising results in vitro and in vivo. Yet, clinical data is scarce and partially controversial: By systemic administration of Dovitinib biologically active concentrations could be consistently achieved in 13 patients with NMIBC. However, long-term administration was not possible due to frequent toxicities. In another study Dovitinib showed a better tolerability but the antineoplastic effect in patients with FGFR3-mutated and FGFR3 wild type urothelial bladder cancer was poor. For AZD4547 a case of long term response is described. BGI-398 showed an overall response rate of 36% in patients with pretreated advanced or metastatic urothelial carcinoma and was well tolerated.

Besides a low FGFR3 expression, CDKN2A-high tumours also showed a low ESR2 expression and AR was negatively correlated with CDKN2A. CDKN2A expression was positively correlated with PDCD1, CD274 and CTLA4 expression. Yet, this did not result in a differential expression in CDKN2A-high tumours. In general the expression of the tested drug target genes was rather low in CDKN2A-high tumours. Therefore they may represent a high risk population both in terms of prognosis and limited treatment options.

Correlation of CDKN2A with the downstream markers CDK4, RB1 and E2F3 in the TCGA cohort also point to a more complex role of CDKN2A in the biology of MIBC. Unlike to be assumed by the known mechanism of CDKN2A-CDK4 interaction, with CDKN2A typically deactivating CDK4, the expression of both genes is not inversely but positively correlated. Furthermore, the tumour suppressor RB1, which is the subsequent gene in this signaling cascade, is significantly downregulated upon increasing CDKN2A expression, indicating a more active cell cycle despite a high CDKN2A expression. Since the P16 protein mainly functionally regulates downstream targets via binding of CDK4 and CDK6, preventing them from interaction with cyclin D, which then results in reduced phosphorylation of RB1, phosphorylation data of RB1 would offer a more precise information about the pathway activity downstream of P16. Yet, there are currently no larger datasets analyzing RB1 phosphorylation status in bladder cancer.

The 2014 TCGA publication, comprising data from 131 MIBC, described four different subtypes based on RNA expression data. These subtypes are mainly determined by the expression of luminal cytokeratins KRT8 and KRT18, basal cytokeratins KRT5, KRT16A, KRT6B, KRT6C, KRT15, the transcription factors GATA3 and FOXA1 and uroplakins. The updated 2017 publication suggests five molecular subtypes and implemented elements in the biology of MIBC: Unlike to be assumed by the known mechanism of CDKN2A to a more complex role of CDKN2A-high tumours. The updated 2017 publication suggests five molecular subtypes and implemented elements in the biology of MIBC. Unlike to be assumed by the known mechanism of CDKN2A.

Methods

Study population, RNA isolation and qRT-PCR. The test cohort consisted of 80 patients (mean age 66 years, range 46–93 years) with MIBC who underwent radical cystectomy at the Mannheim University Hospital between January 1998 and December 2006. Clinical and pathological data were retrospectively obtained from medical records (ethics approval 2016-814R-MA of the medical ethics committee II of the medical faculty of the University of Heidelberg).

RNA extraction was performed as described before. 10 µm sections from FFPE tissue samples were used for RNA extraction with a commercially available bead-bound extraction method (XTRACT kit; STRATIFYER Molecular Pathology GmbH, Cologne, Germany). RNA was eluted with nuclease-free water (Life Technologies) and hydrolysis probes were diluted to 100 µM, using a stock solution with nuclease-free water (Life Technologies).
status and RNA-expression subtypes. Kruskal-Wallis test with post hoc Dunn’s test for multiple comparisons was performed on a stratified patient cohort and the TCGA cohort. For the analysis of expression according to CDKN2A, statistically significant.

expression groups in the TCGA cohort was downloaded from Gene Expression Omnibus (GSE48276). Cancer Genome Atlas) project served for outcome validation. Illumina array RNA expression data of the MDA cohort was downloaded from Gene Expression Omnibus (GSE48276).

Statistics. Statistical analyses were performed using SAS JMP version 11.0 (SAS Institute, Cary, NC, USA) and GraphPad PRISM (Version 7.0; Graph Pad Software Inc., La Jolla, CA, USA). Cut-Off definitions were done by partitioning tests for decision trees to determine different CDKN2A expression groups. Student’s t-test and Chi² test were used to compare for differences in the distribution of clinical parameters, TCGA subtypes and CDKN2A CNV data between the CDKN2A expression groups.

Kaplan Meier analyses were performed for DSS and RFS in the test cohort and for DSS and OS in the validation cohorts and were tested for significance using Log-Rank test.

Both in the test cohort and in the TCGA cohort CDKN2A expression was correlated with FGFR3 expression using Spearman correlation. In the TCGA cohort CDKN2A expression was also correlated with the expression of AR, ERα, ERβ2, PDCD1, CD274, CTLA4, KRT5, KRT6A, KRT6B, KRT6C, KRT8, KRT14, KRT18, UPK1A, UPK1B, UPK2, UPK3A, UPK3B, GATA3, FOXA1, CDK4, RB1, E2F2 and K67.

Student’s t-test was used to test for differences in gene expression in the CDKN2A expression groups in the test cohort and the TCGA cohort. For the analysis of CDKN2A expression according to CDKN2A copy number status and RNA-expression subtypes Kruskal-Wallis test with post hoc Dunn’s test for multiple comparisons was performed in the TCGA cohort. Graphs were designed with Graphpad Prism. P-values < 0.05 were deemed statistically significant.

Ethical approval. 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. Due to its retrospective character, for this type of study formal consent is not required.

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Author Contributions
R.S., W.O., A.H., C.B., R.M.W. and P.E. planned the study and the experiments. T.S.W., C.A.W. and P.E. assembled the test cohort. C.A.W., S.B., R.S., M.E. and A.H. performed pathologic evaluation of tissue sections. R.M.W. performed PCR analyses. T.S.W., A.H., R.M.W. and P.E. performed PCR data analysis. T.S.W., A.H., R.M.W. and P.E. performed analyses of the TCGA cohort. T.S.W., J.B., A.H. and P.E. wrote the manuscript. All authors reviewed the manuscript.

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