Peer Review File

Article information: https://dx.doi.org/10.21037/tau-22-494

Reviewer A

Authors Jason Zhu and colleagues have pursued to verify if there is a relationship between Galectin (Gal)-1 and Gal-3 expression, in a large cohort of patients who underwent cystectomy in both non-muscle and muscle-invasive bladder cancer and if the expression levels have a relationship with respect to overall survival and recurrence-free survival. The authors have used immunostaining on tissue microarrays generated from 32 tumor blocks from chemotherapy naïve cystectomy specimens for the expression of these two proteins. The study is well-designed with enough numbers available for the Robustness of the data. The data is well presented and organized. The manuscript should be acceptable for publication if the authors can address the following issue.

1. As the results of the present study differ from prior studies which have described a relationship between galectin expression and overall survival in patients with bladder cancer, it is appropriate to discuss the difference in the methodology used in the current study compared to the previous study the authors are referring the details of both the antibodies used for immunostaining are available, however, it would be appropriate to quote other studies that have used the same antibody for immunostaining.

Reply 1: Thank you for this comment. I have added citations which have used the M3/38 and C-8 antibody.
Changes in the text: Citations added line 100-101

2. Also, for normalization of immunostaining any housekeeping protein levels were evaluated. If not, why not? especially given the heterogeneity within the tumor

Reply 2: While it would be optimal to assess for normalization of immunostaining, there is limitation on the tissue for this tissue microarray that was created at another institution. We do not have the ability to test for housekeeping protein levels. This TMA has been previously used for other studies with expected staining pattern.
Changes in the text: n/a

This is a well-designed study. Minor limitations in the methodology have been pointed out. The sample size makes it one of the largest studies in this cancer group, so with minor changes the information from the study will be more information for researchers in this field of study.

Reviewer B

1) General comments

Using immunohistochemistry for tissue microarray (TMA) from total cystectomy specimens, Zhu et al. analyzed Galectin (Gal)-1 and Gal-3 expression in the urinary bladder urothelial carcinomas, and failed to reveal significantly different expression between non-muscle invasive bladder cancers (NMIBCs) and muscle invasive bladder cancers (MIBCs), and its impact in patient's prognosis. Although this topic is interesting, there are major flows in research methods, such as lack of locoregional data of invasive cancers (distinction of T1, T2, T3, and T4) in TMA cores and lack of information how to perform immunohistochemical H scoring. In addition, description of Results is sometimes inaccurate and needs to be properly explained in the manuscript. Unless the correction of these point, the authors' data do not logically connect to their conclusion.

Reply 1: Thank you for your comprehensive comments. We agree, the lack of granularity between the T stages for the "invasive" tissues is a major limitation of this study. At the time of the creation of this tissue microarray, there was no distinction made between T1-T4 and all were categorized in one cohort.

2) Specific comments

a) major

1. One of the main purposes of this study is a comparison of Gal-1/Gal-3 expressions in NMIBCs with those in MIBCs (The reviewer interpreted "invasive cohort" and "non-invasive cohort" in Tables 5-6 as "MIBCs" and "NMIBCs", respectively). However, as described in Discussion, invasive levels (i.e., T1, T2, T3, or T4) of TMA cores were not distinguishable. Because of heterogeneity of the tumor (as the author also indicated), Gal-1/Gal-3 expression in MIBCs should be investigated in muscle-invasive cancer cells. Whole slide analysis of Gal-1/Gal-3 expression in at least a subset of the research cohort is required to assess this issue.

Reply 2: Unfortunately, we do not have access to the original tissue blocks for whole slide immunohistochemistry. The TMA was created at another institution. Multiple punches were taken from each tumor to assess for heterogeneity.

2. Analytical methods of immunohistochemical H score should be briefly described in the manuscript. "Product of the percentage and intensity score" is insufficient information to reproduce the research. The representative microscopic photos of Ga1-1/Gal-3 expression in bladder cancers also need to be represented.

Reply 1:

1. **We utilized the h score method similar to prior galectin studies (Decreased expression of galectin-3 predicts tumour recurrence in pTa bladder cancer Kramer et al, Oncology Reports 2008, Diagnostic and Prognostic Role of Galectin 3 Expression in Cutaneous Melanoma, Gaber et al AJD 2010)**
2. **Representative microscopic photos of Gal-1/Gal-3 have been added in the figures**

Changes in the text:

1. **Added figure 1 which provides representative microscopic photos of Gal-1 and Gal-3**

3. Some of clinicopathological variables including Charlson Comorbidity Index (CCI) and The American Society of Anesthesiologists (ASA) class need references.

ASA score is not written in the manuscript (only in Tables).

CCI seems to be better to be represented as discrete data (instead of continuous data) such as 1-2, 3-4, and 5 or more, in Cox regression analysis, as same as ASA class.

Serum albumin is included in the variables in the manuscript, however, there is no data in Tables.

There is no data of positive nodes in Table 2.

In addition, not only multivariable analysis, but univariate Cox analysis should be performed and described in the manuscript and Tables.

Partial lack of clinicopathological information (e.g. number of nodes is available in 297 of 301 cases) need to be presented in the manuscript.

Reply 3: Thank you for these comments, we agree and have made the following changes in our text as noted below.

Changes in the text:

1. **References for American Society of Anesthesiologists, Charlson Comorbidity Index, and Staging added in lines 114-116**
2. **Added ASA score in manuscript**
3. **On table 2, node status is on listed under section Pathological N stage**
4. **Added statement reference lack of node status in 4 of the 301 cystectomy specimens in line 132-133 and corrected the calculation of patients from 23% to 24%**

5. **CCI was changed from continuous data to discrete data in table 1**
6. **Serum albumin has been updated in table 1. However, we did not include this in the model as many patients (30%) were missing this variable and would significantly decrease the sample size**
7. **Univariate cox analysis was added to table 3**

4. Lines 119 and 217: The reviewer does not understand meaning of "most invasive tissue type" and "the most invasive area of the tumor".

Reply 4: Given that many patients had cores of different T stages of their specimen, we took the H score from the highest T stage. I will change this in the text to make it more clear
Changes in the text:

1. **Changed lines 120-121 to and replaced "most invasive" with highest T stage**
2. **Changed line 214 to highest T stage as well**

5. To use an abbreviation, write out the term or phrase on first use, and after that, all terms should be abbreviated in the manuscript: not mixture of full spelled and abbreviated terms (e.g. Gal-1 and galectin-1). There are numerous disunities of the abbreviations in the whole manuscript. Terms in abstract should be separately abbreviated from those in main manuscript.

Reply 5: Thank you for this comment, the paper will be edited in concordance with this guideline
Changes in the text: Multiple changes in the document to comply with this guideline with regards to abbreviations Gal-1, Gal-3, OS, RFS, MIBC, NMIBC

6. Line 142: "preoperative packed cell volume" is not significantly associated with overall survival; p value is 0.051 in Table 3.

Reply 6: Agreed
Changes in the text: Changed line 142

b) Minor:
There are minor but many inaccurate descriptions and typos in the manuscript. A part of those is indicated below:
1. Lines 76 and 77, "elevated expression levels were associated overall survival" does not make sense; "shorter" or "longer" should be added.
2. The reviewer do not understand the meaning of the sentence in lines 122-124.
3. Line 152: "Cis" should be "Tis".

4. Line 198: there is grammatical error: “differences between our studies may differ...”.
5. The sentence in lines 199-202 needs appropriate reference(s).
6. Line 271: “progression free survival” should be “RFS”.

Reply 6B:

1. **Agreed, corrections made**
2. **Pending**
3. **Agreed, corrected**
4. **Agreed, corrected**
5. **Citations added**
6. **Agreed, corrected**

Reviewer C

1. The paper is interesting, however, most of the tables included in the main text make it hard to follow and confusing. In my opinion, the body of the results section should contain only the most important data and the other methodological data should be moved to the supplementary materials sections and referred to in the main text.

Reply 1: Thank you for this comment.

2. The lack of differences in the overall survival in patients expressing galectins may result from the fact that you analyze the group as the whole. Maybe the Authors should divide the group into different tissue classes?

Reply 2: Thank you for this comment. We recognize that bladder cancer patients are a heterogenous population, especially given the differences in survival between patients with muscle invasive disease and non-muscle invasive disease. Thus, separated the analysis for these two cohorts, as described in lines 146-149.

3. Data concerning the survival of the patients with respect to lymph node status does not concern the expression of galectins. Maybe the analysis dividing the patients into groups according to lymph nodes status would reveal the prognostic significance of galectins? Otherwise, an interesting paper. The paper is interesting, however, I would recommend the authors to do some more analysis of the data

Reply 3: Thank you for this comment. **We included lymph node status in our multivariable analysis. After controlling for lymph nodes and other variables, Gal-1 and Gal-3 were not associated with overall survival.**