SAM68 is a useful prognostic factor for muscle invasive bladder cancer and
associated with the progression of non-muscle invasive bladder cancer

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Keywords: SAM68; bladder cancer; prognosis; biomarker; progression.
Abstract

**Background:** Muscle invasive bladder cancer (MIBC), one kind of lethal disease, still lacks effective prognostic marker. Non-MIBC (NMIBC) presents high rate of recurrence and potential risk of progression. SAM68, a member of RNA-binding proteins, was reported played an important role in cancer progression. The aim of this study was to investigate the importance of SAM68 in MIBC prognosis. We also studied the role of SAM68 in NMIBC progression.

**Methods:** Quantitative PCR and immunohistochemistry were utilized to detect the expression of SAM68 in ten pairs of MIBC tissues and adjacent normal bladder urothelial tissues, and also in eight pairs of NMIBC and MIBC tissues from the same patient. Moreover, SAM68 protein expression level and localization in 129 clinicopathologically characterized MIBC samples were examined by immunohistochemistry. Prognostic associations were examined by statistical analyses.

**Results:** SAM68 expression was elevated in human MIBC tissues compared with adjacent normal bladder urothelial tissues. High expression and nucleus-cytoplasm co-expression of SAM68 were associated with higher T stage, higher N stage and worse recurrence free survival. The low SAM68 expression group had a cumulative 5-year recurrence free survival rate of 80.0%, whereas the high SAM68 expression group only had 52.9% (p=0.001). Patients with Sam68 nucleus-cytoplasm co-expression have a worse recurrence free survival (49.2%) than those with Sam68 nucleus (82.5%) or cytoplasm (75.5%) alone. Multivariate analysis revealed that SAM68 expression level, nucleus-cytoplasm co-expression, as well as T and N stage, were recognized as independent prognostic factors for the recurrence free survival of MIBC patient. More interestingly, SAM68 expression increased at both transcriptional and translational levels in MIBC tissues compared with NMIBC tissues of the same patient.

**Conclusions:** SAM68 is a useful prognostic factor for MIBC and may also be involved in the progression of NMIBC.
Background
Bladder cancer is the fourth most-common malignancy in men after prostate, lung, and colorectal cancers, accounting for 7% of all cancer cases. It is reported in the Cancer Statistics that there will be 74,690 new cases of bladder cancer and 15,580 cancer-related deaths in the USA in 2014[1]. About 75% of bladder cancer presented as non–muscle invasive tumors (NMIBC)[2]. For this kind of early stage bladder cancer, about 50%-80% patients will had tumor recurrence after transurethral resection of bladder tumor (TURBT). What's worse is that about 50% T1 tumors will had tumor progressed to muscle invasive bladder cancer (MIBC) [3]. Another 25% bladder cancer patients are diagnosed as muscle invasive disease at their first visit to doctors[4-5]. Unfortunately, the 5-year survival rate for patients with invasive bladder cancer is still low, about 33% for regional and 5.4% for distant-stage disease[2]. Around 1/3 patients will suffer cancer recurrence after cystectomy even with adjuvant chemotherapy [6]. Although altered expression of oncogenes and tumor suppressors has been found in bladder cancer[7], the power of many well identified biomarkers in predicting the clinical outcome of individual tumors is limited due to the great heterogeneity of this cancer. Therefore, the discovery of alternative biomarkers involved in the development and progression of bladder cancer may lead to the identification of new prognostic markers and therapeutic targets.

SAM68 (Src-associated in mitosis, 68 kDa) is a member of the STAR (signal transduction and activation of RNA) family of RNA-binding proteins[8]. SAM68 protein had an hnRNP K homology domain (KH domain) that locates within a larger GSG (GRP33-SAM68-GLD1) domain that is required for specificity and high-affinity binding to RNA[8-9]. This multimodular structure allows SAM68 protein to exert different functions in the cell. It is reported that SAM68 plays important role in cell cycle, cell proliferation, and apoptosis. SAM68 is also associated with many kinds of cancer progression and/or prognosis, including renal cell carcinoma[10], breast cancer[11], cervical cancer[12], prostate cancer[13], etc. However, the relationship between SAM68 expression and bladder cancer prognosis and progression has never been reported.
In the current study, we reported for the first time the characterization of SAM68 expression and localization in human MIBC tissues, and its correlation with clinicopathological characters of this disease. We also studied the role of SAM68 in NMIBC progression. By doing so, we aimed at finding a novel and useful marker for the prognosis of MIBC and progression of NMBIC.
Materials and Methods

Patients and tissue specimens

Ten pairs of MIBC tissue specimens and corresponding non-tumorous specimens were obtained from patients with bladder cancer who underwent radical cystectomy at the Cancer Center of the Sun Yat-sen University (Guangzhou, P. R. China). Eight paired of NMIBC and MIBC tissues were obtained from two operations of the same patient. The NMIBC tissue obtained from TURBT and paired MIBC tissue obtained from radical cystectomy. All excised tissues were obtained within 1 h after surgery and were immediately placed in liquid nitrogen until further analysis.

Immunohistochemistry analyses were performed on 129 paraffin-embedded radical cystectomy samples, which were histologically diagnosed as MIBC at the Cancer Center, Sun Yat-sen University, between 2000 and 2008. Patient consent and approval from the Institutional Research Ethics Committee were obtained for the use of these clinical materials for research purposes. Tumor-node-metastasis (TNM) staging was determined according to the 2010 American Joint Committee on Cancer TNM classification of bladder cancer[14]. The detail of patients’ information are summarized in Table 1. The median follow-up period for this cohort of patients was 32 months (range, 6-104 months). During the follow-up period, 35 patients had tumor recurrence.

RNA extraction and quantitative PCR

Total RNA from tumor and adjacent non-tumorous tissues was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) was performed according to standard methods as described previously [10]. PCR primers and probes were designed with the use of Primer Express Software v.2.0 (Applied Biosystems) as described previously [10].

Immunohistochemistry

Immunohistochemistry (IHC) was performed to study altered SAM68 protein expression levels in 129 human MIBC tissues, as well as the ten pairs of MIBC tissue specimens and corresponding non-tumorous specimens, and eight paired of NMIBC and MIBC tissues. In brief, 4 μm-thick tissue sections were incubated with polyclonal
rabbit antibody against SAM68 (1:200; Abgent) at 4°C overnight. Before incubation
with the primary antibody, the sections were treated for antigen retrieval with ethylene
diamine tetraacetic acid buffer followed by heating in a microwave oven. For negative
controls, the rabbit anti-SAM68 antibody was restored with normal nonimmune serum.
After washing, tissue pieces were treated with biotinylated anti-rabbit secondary
antibody (Zymed), followed by further incubation with streptavidin -horseradish
peroxidase complex (Zymed). Tissue sections were then immersed in
3,3′-diaminobenzidine and counterstained with 10% Mayer's hematoxylin, dehydrated,
and mounted.

The degree of immunostaining of paraffin-embedded sections was reviewed and
scored independently by two observers based on the proportion of positively-stained
tumor cells and the intensity of staining. The method has been introduced in detail
previously[10]. The staining index was calculated as the product of the staining
intensity score and the proportion of positive tumor cells. Using this method of
assessment, we evaluated SAM68 expression in MIBC tissues by determining the
staining index, with scores of 0, 1, 2, 3, 4, 6, 8, 9, and 12. SAM68 threshold values
were chosen on the basis of a measure of heterogeneity with the log-rank test
statistical analysis with respect to recurrence free survival. An optimal threshold value
was identified: a staining index score >6 was considered to show high SAM68
expression, whereas a staining index score <4 was considered to represent low
SAM68 expression.

Statistical analysis

The relationship between SAM68 expression and clinicopathological
characteristics was analyzed by the chi-square and Fisher’s exact tests. Survival
curves between subgroups that were divided according to SAM68 expression levels
and localizations were drawn with the use of the Kaplan-Meier method, and
significant differences among subgroups were compared by the log-rank test. Survival
data were evaluated using univariate and multivariate Cox regression analyses. In all
cases, P < 0.05 was considered to be statistically significant. All statistical analyses
were conducted using the SPSS v.13.0 statistical software package (SPSS, Chicago,
IL, USA).
Results

The expression of SAM68 in paired of MIBC tissues and adjacent normal bladder urothelial

To determine the expression of SAM68 in MIBC tissues, quantitative PCR analyses were conducted with ten matched pairs of bladder cancer tissues and adjacent, noncancerous bladder urothelial tissues from the same patient. As shown in Figure 1, all the ten bladder cancer showed up-regulated SAM68 mRNA expression, when compared with their adjacent bladder urothelial tissues. IHC analysis further confirmed these results, in the translation level (Fig 1B). This data suggest that SAM68 expression was elevated in human MIBC.

The expression of SAM68 in 129 MIBC tissues from radical cystectomy

To study the clinicopathological significance of SAM68 in MIBC, we examined the SAM68 protein expression levels in 129 paraffin-embedded, archived MIBC, from radical cystectomy, with long term follow-up data. The IHC results are summarized in Table 1. SAM68 protein was detected in 123 out of 129 (95.3%) human MIBC samples, in which 70(54.3%) showed high expression of SAM68 and 59(45.7%) showed low expression of SAM68 protein (Fig 2A). For the localization of SAM68 protein, there were three types of expression pattern, including nucleus expression, cytoplasm expression and nucleus-cytoplasm co-expression (Fig 2B). 43 cases (35.0%) had SAM68 expression in nucleus, 23 cases (17.8%) in cytoplasm and 57 cases (46.3%) had SAM68 expressed in both nucleus and cytoplasm.

High expression and nucleus-cytoplasm co-expression of SAM68 were associated with clinical features and recurrence free survival

To determine the association of SAM68 expression and clinical features and recurrence free survival of MIBC, \( \chi^2 \) test and survival analysis with long-rank test were performed. As shown in Table 1, high expression and nucleus-cytoplasm co-expression of SAM68 were correlated with the T stage (p=0.028, and p=0.035) and N stage (p=0.041, and p=0.050). The Kaplan-Meier survival analysis indicated that patients who had high SAM68 expression levels had worse recurrence free survival than those with low SAM68 expression. The low SAM68 expression group had a
cumulative 5-year recurrence free survival rate of 80.0% (95% confidence interval [CI], 0.680-0.919), whereas the high SAM68 expression group only had 52.9% (95% CI, 0.384-0.674). (Fig 3A). Patients with SAM68 nucleus-cytoplasm co-expression had lower 5-year recurrence free survival rate (49.2%) than those with SAM68 expressed in nucleus (82.5%) or cytoplasm (75.5%). However, the difference of recurrence free survival curve between SAM68 nucleus group and cytoplasm group is not significant (Fig 3B). In addition, the multivariate analysis revealed that SAM68 expression level, nucleus-cytoplasm co-expression, accompanied with T and N stage, were recognized as independent prognostic factors for the recurrence free survival rate of MIBC patient (Table 2). These results indicated that SAM68 could be a valuable prognostic marker for MIBC after radical cystectomy.

The expression of SAM68 in paired of NMIBC and MIBC tissues

To explore the role of SAM68 in bladder cancer progression, we analyzed eight MIBC patients with a history of non–muscle invasive bladder tumor, who underwent radical cystectomy for recurrent tumor infiltrated into or beyond muscularis propria. Paired of NMIBC and MIBC were obtained from TURBT and radical cystectomy, respectively, of the same patient. The results indicated that SAM68 was markedly up-regulated at both transcriptional and translational levels in MIBC tissues compared with NMIBC tissues by quantitative PCR and IHC (Fig 3A and 3B). These findings suggested that bladder cancer developing from NMIBC to MIBC is accompanied with SAM68 expression level ascending.
Discussion

MIBC is one kind of excruciating disease requiring radical cystectomy as golden standard treatment[15]. After radical cystectomy, the quality of life greatly reduced for the accompanying of incontinence, impotence and abdominal wall storm, etc[16-17]. Even worse, the long term survival rate is not satisfying after radical cystectomy, especially for disease infiltrated into the perivesical tissues[15, 18]. All this characters make finding a useful prognostic marker for this disease as a urgent. All this time, scholars try to find some useful biomarkers which can provide prognostic information for bladder cancer. Margulis and colleagues[19] found that Ki-67, a marker for proliferation, may be a prognostic marker for bladder cancer patients after radical cystectomy. They reported a high Ki-67 labeling index was independently associated with disease recurrence and cancer-specific survival of bladder cancer patients. Another meta-analysis[20] pooled the results of 16 studies which investigated the role of p53 in predicting the prognosis of bladder cancer. They reported a overall hazard ratio of 1.43 for p53 to predict mortality. However, due to the complicated biological behavior of bladder cancer, none of these markers have been widely used in clinical practice. Hence, it is still necessary to find some novel predictive factors for bladder cancer. In the current study, we reported for the first time SAM68 as a potential prognostic marker for MIBC. In this cohort, 54.3% patients showed high expression of SAM68 protein. Patients with high expression of SAM68 in tissue sample have 2.4 fold higher risk of disease relapse than those with low SAM68 expression. These results suggesting SAM68 can act as a candidate of prognostic factor for MIBC. Further study with lager sample validating the role of SAM68 in MIBC is worthy.

The role of SAM68 in cancer development and progression is controversial to some extent, although most studies identified SAM69 as a tumor-promoter[10-13, 21]. In our study, we found the expression level of SAM68 is much higher in MIBC tissues than adjacent normal bladder urothelial tissues. High expression of SAM68 was associated with worse clinicopathologic parameters and prognosis. Moreover, SAM68 expression level ascending accompanied bladder cancer developing from NMIBC to MIBC. Our research supported SAM68 as a tumor-promoter in MIBC.
However, the reason why high SAM68 expression is associated with bladder cancer prognosis is not clear. In some other cancers, the function and mechanism of SAM68 in cancer development and progression has been revealed to some degree. In prostate cancer, it has been reported that a subset of genes involved in proliferation and apoptosis were altered when SAM68 was knocked down in LNCaP cells, including Bcl2L1, Clusterin, cdk2, cdk3, p16INK4, cyclin D1, Par-4, EGF and IGF-1 [22]. Song et al reported that silencing Sam68 can cause anti-proliferative effect on breast cancer cells. The possible mechanism may be that silencing Sam68 can up-regulate p21 and p27, and attenuation of Akt/GSK-3β signalling [11]. Our finding not only supported that SAM68 plays as a predictor for bladder cancer prognosis and progression, but also warrant further studies on the relationship between SAM68 and bladder cancer biological behavior.

We also found 46.3% cases had SAM68 expressed both in nucleus and cytoplasm, which we called as nucleus-cytoplasm co-expression. This expression pattern is also associated with T and N stage and recurrence free survival. Furthermore, SAM68 nucleus-cytoplasm co-expression is an independent poor prognostic of the recurrence free survival for bladder cancer patients. Although SAM68 was initially reported predominantly localizes in the nucleus [23-24], SAM68 cytoplasmic localization can be observed in several human cancers and had clinical significant, including breast cancer and early-stage cervical cancer [11-12]. Although the function and mechanism of SAM68 accumulation in the cytoplasm remain unknown, several hypotheses have been proposed. It has been demonstrated that SAM68 is localized in the cytoplasm and associated with the polysomes that are engaged in active messenger RNA (mRNA) translation [22, 25]. SAM68 could associate with polysomal mRNAs in the cytoplasm and enhance the translational efficiency of the translation elongation factor, eEF1, which has been implicated in cell transformation [26-27]. It is particularly noteworthy in our study that the prognosis is much poorer in nucleus and cytoplasm co-expression group compared with either nucleus or cytoplasm group. However, the prognosis of SAM68 nucleus group and cytoplasm group is similar. A reasonable explanation for this phenomenon might be
that SAM68 cause poor prognosis of bladder cancer by pulling two triggers in both
nucleus and cytoplasm simultaneously. Only one trigger pulled either in nucleus or
cytoplasm may not lead to more aggressive behavior of bladder cancer cells. However,
the definite function and mechanism of SAM68 accumulation both in the nucleus and
cytoplasm and its correlation with bladder cancer prognosis require further study.

Although our study enrolled a large group of muscle invasive bladder cancer
patients, there are still some limitations in our study. Firstly, this was a retrospective
study, and some bias is therefore inevitable. Secondly, the mechanism and function of
the accumulation of SAM68 in the cytoplasm did not been investigated in this study.
Thirdly, the role of high expression of SAM68 in bladder cancer cell invasion has not
been illuminate. Finally, the association between SAM68 and NMIBC progression
was concluded based on small sample observation. Hence large series validation is
needed in future studies.

Conclusions

In summary, our study suggested SAM68 as a potential prognostic marker for
MIBC. We also preliminarily revealed the relationship between SAM68 and NMIBC
progression. These results not only enrich the investigation of the role of SAM68 in
human cancer, but also make SAM68 a useful biological marker in the prognosis and
progression of bladder cancer.
Abbreviations:

MIBC: muscle invasive bladder cancer
NMIBC: non-muscle invasive bladder cancer
TURBT: transurethral resection of bladder tumor
TNM: Tumor-node-metastasis
PCR: polymerase chain reaction
IHC: immunohistochemistry

Competing interests:
The authors declare that they have no competing interests.

Authors' contributions:
ZZL and YCP performed the IHC and drafted the figure, tables and manuscript.
JLJ and YCP performed the PCR and statistical analyses. LYH and ZZL collected the tissue specimens, patient information and follow-up data. ZFJ supervised the whole study and edited the manuscript. All of the authors read and approved the final manuscript.

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Figure Legends

Figure 1. Expression of SAM68 mRNA and protein in paired muscle invasive bladder cancer tissues and adjacent non-carcinoma tissues. (A) Quantitative PCR showed the mRNA level of SAM68 was much higher in bladder cancer tissues compared with adjacent non-carcinoma tissues. Expression levels were normalized for GAPDH. Error bars represent the standard deviation (SD) calculated from three parallel experiments. (B) Immunohistochemistry analysis was performed to detect the SAM68 protein level in tumor (T) and non-carcinoma tissues (N) from the same patient.

Figure 2. Typical images showed the expression level and localization of SAM68 protein as examined by immunohistochemistry (IHC). (A) Series showed the negative, low level and high level expression of SAM68 protein. Left panel: 200×; right panel: 400×. (B) Three types of SAM68 expression according to the intracellular localization: nucleus expression, cytoplasm expression and nucleus-cytoplasm co-expression. Left panel: 400×; right panel: 800×.

Figure 3. Kaplan-Meier curves with log-rank test comparing recurrent free survival (RFS) in different subgroups. (A) Bladder cancer patients with low SAM68 expression (bold line) had a cumulative 5-year RFS rate of 80.0%, compared to 52.9% for patients with high SAM68 expression (dotted line; p=0.001). (B) Comparison of RFS curves between groups with different SAM68 intracellular localization. The RFS curves were similar between SAM68 nucleus expression and cytoplasm expression groups (p=0.539). However, the RFS was much poorer in the nucleus-cytoplasm co-expression group, no matter compared with nucleus expression group (p=0.002) or cytoplasm expression group (p=0.037).

Figure 4. Expression of SAM68 protein was increased when bladder tumor developed form non-muscle invasive to muscle invasive. (A) Increased fold of SAM68 mRNA for muscle invasive tumor comparing to non-muscle invasive tumor from the same patient. (B) Immunohistochemistry study confirmed the expression of SAM68, in the protein level, was much higher in muscle invasive tumor samples than non-muscle invasive tumor from the same patient. For each pair of sample, the left panel represents non-muscle invasive bladder cancer (NMIBC) sample obtained from transurethral resection of bladder tumor and the right panel represents muscle invasive bladder cancer (MIBC) sample gained from radical cystectomy.
Table 1: Correlation between clinicopathological features and SAM68 expression in MIBC patients.

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<td>26(66.7)</td>
<td>0.083</td>
<td>37</td>
<td>13(35.1)</td>
<td>4(10.8)</td>
<td>20(54.1)</td>
</tr>
<tr>
<td>No</td>
<td>90</td>
<td>46(51.1)</td>
<td>44(48.9)</td>
<td></td>
<td>86</td>
<td>30(34.9)</td>
<td>19(22.1)</td>
<td>37(43.0)</td>
</tr>
</tbody>
</table>

MIBC: muscle invasive bladder cancer; UIS: urinary irritation symptoms
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>pT stage (T3~4 vs. T2)</td>
<td>4.445</td>
<td>2.221~8.896</td>
</tr>
<tr>
<td>pN stage (N+ vs. N-)</td>
<td>4.944</td>
<td>2.313~10.780</td>
</tr>
<tr>
<td>SAM68 level (High vs. low)</td>
<td>3.366</td>
<td>1.574~7.201</td>
</tr>
<tr>
<td>SAM68 Localization*</td>
<td>2.003</td>
<td>1.267~3.166</td>
</tr>
</tbody>
</table>

MIBC: muscle invasive bladder cancer; HR: hazard ratio; CI, confidence interval;
*nucleus-cytoplasm co-expression vs. expression in nucleus vs. expression in cytoplasm
Figure 1

(A) Bar graph showing increasing fold changes for samples 1 to 10, with error bars indicating variability. Samples 3, 4, 5, 6, 7, 8, 9, and 10 are labeled as N (normal) and T (treated). Samples 1 and 2 are not labeled.

(B) Images of histological sections showing the expression levels of the proteins of interest, with N (normal) and T (treated) tissues compared.
Figure 2

A

200X  400X

Negative

Low expression

High expression

B

400X  800X

Nucleus expression

Cytoplasm expression

Nucleus-cytoplasm co-expression
Figure 4

A

![Bar graph showing increasing fold values for samples 1 to 8](image)

B

- **Sample 1**
  - NMIBC vs MIBC
  - Images showing tissue samples

- **Sample 2**
  - NMIBC vs MIBC
  - Images showing tissue samples

- **Sample 3**
  - NMIBC vs MIBC
  - Images showing tissue samples

- **Sample 4**
  - NMIBC vs MIBC
  - Images showing tissue samples

- **Sample 5**
  - NMIBC vs MIBC
  - Images showing tissue samples

- **Sample 6**
  - NMIBC vs MIBC
  - Images showing tissue samples

- **Sample 7**
  - NMIBC vs MIBC
  - Images showing tissue samples

- **Sample 8**
  - NMIBC vs MIBC
  - Images showing tissue samples