STAT1 and STAT4 expression as prognostic biomarkers in patients with bladder cancer

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Received August 16, 2024; Accepted December 11, 2024

DOI: 10.3892/mco.2025.2828

Abstract. Signal transducer and activator of transcription (STAT) proteins are cytoplasmic transcription factors known to play key roles in numerous physiological and pathological processes, from pathogen response to cancer modulation. However, the roles of some STAT family members, particularly STAT1 and STAT4, in the initiation and progression of bladder cancer (BC) have not been comprehensively studied. The present study investigated the expression pattern of STAT1 and STAT4 in the prognosis and survival of BC taking advantage of patients' specimens and cell lines. In our cohort, high mRNA expression of STAT1 was significantly associated with tumor invasiveness, recurrence and progression, and was shown to increase according to tumor stage in BC cell lines. However, it did not affect patient survival. By contrast, STAT4 exhibited its highest expression in early-stage tumors, without a significant link to the tumor stage. Moreover, it was found that increased STAT4 mRNA expression was associated with improved disease-free survival and overall survival in our cohort. Collectively, these findings suggest that STAT1 and STAT4 could be promising prognostic markers to enhance BC management.

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Key words: STAT1, STAT4, bladder cancer, prognosis marker, survival

Introduction

The molecular characterization of bladder cancer (BC) has transformed understanding of BC pathogenesis and paved the way for new biomarker discoveries. However, translating these insights into clinical practice remains a challenge due to the complexity of the disease and the multitude of altered molecular and pathological pathways (1,2).

Signal transducers and activators of transcription (STAT) proteins are key players in influencing tumor behavior and modulating the tumor microenvironment. The STAT family is composed of seven transcription factors (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6), which play a critical and multifaceted role in regulating vital physiological processes such as cell proliferation, differentiation, apoptosis, angiogenesis and the epigenetic organization of immune cells. Beyond these functions, STATs also contribute to pathological processes by selectively inducing and maintaining a pro-carcinogenic inflammatory microenvironment at the initiation of malignant transformation and during cancer progression (3-6). Dysregulation in this pathway is associated with poor prognosis in various cancers and has been recognized as a common driver in BC, promoting tumor cell proliferation and motility (7).

STAT1 is considered a tumor suppressor, although there is increasing evidence for its tumor-promoting functions (8). The depletion of STAT1 in cancer cells, observed in a broad spectrum of cancers, is often correlated with a poor prognosis. However, the mechanism behind the oncogenic or tumor-suppressing role of STAT1 remains unclear (9). STAT4 is involved in carcinogenesis and tumor progression (6). Although STAT4 inhibition appears to promote tumorigenesis and predict poor outcomes in hepatocellular carcinoma and gastric cancer, its expression was reported to be significantly increased in colorectal cancer specimens, and associated with tumor spread, suggesting a pro-tumorigenic role of STAT4 in

this type of cancer (10-12). In ovarian cancer, activation of STAT4 is abundant in epithelial cells and its overexpression was associated with poor outcome and promoted metastasis both *in vivo* and i*n vitro*, suggesting a pro-metastatic function of STAT4 (13).

Previous studies reported that STAT1 and STAT4 exhibited both complementary and opposite roles. These functions appear to be regulated in a manner dependent on both cell type and cancer type (14,15). The molecular events in bladder tumorigenesis have sparked considerable interest, as they hold promise for advancing patient diagnosis, prognosis and the development of targeted therapies. While STAT1 has been extensively studied in BC due to its well-established antitumor role, the involvement of STAT4 in tumor development and progression remains largely unexplored, with only limited insights available.

Considering the significant roles of STAT1 and STAT4 in cancer, as well as the increasing need for improved tools to stratify patients and develop reliable prognostic and diagnostic biomarkers, the expression level of STAT1 and STAT4 was evaluated in primary and recurrent BC patient specimens, spanning the full range of the disease's grades and stages. Their potential associations with clinicopathological features and patient clinical outcomes were also evaluated. Additionally, STAT1/4 expression was quantified in human bladder cell lines mimicking the four stages of tumorigenesis. Overall, the present study provides insight into the expression pattern of STAT1 and STAT4 at the different stages of tumorigenesis and contributes to the identification of potential prognostic biomarkers in BC.

Materials and methods

Characteristics of the patients and tissue samples. A total of 70 fresh frozen tumor samples were obtained by transurethral resection of the bladder or cystoscopy at the Urology Department of the University Military Hospital in Rabat-Morocco between August 2019 and July 2022. The study protocol was approved (approval no. Ref 82/19) by the Ethics Committee for Biomedical Research from the Faculty of Medicine and Pharmacy of Rabat (Rabat, Morocco). Written informed consent was obtained from each recruited patient before sample collection. Staging and grading were conducted according to the World Health Organization Consensus Classification at the Anatomopathology Department of the Military Hospital Mohammed V. The data collected from patients' records is summarized in Table I. Among the 70 recruited patients, 68 men and 2 women were included. The mean age of patients was 67 years, ranging from 47-85 years. A total 28 patients (40%) have reported to be active or former smokers. Most cases were staged ≤ PT1 (74.29%) and had high tumor grade (61.43%). Among patients with non-muscle invasive BC (NMIBC) stage collected during the present study, 12 experienced tumor recurrence (23.08%) and 5 were diagnosed with progression upon the 4-year follow up.

Cell lines and cell culture. A total of four human bladder cell lines were used in the present study: BU68.08, mimicking a NMIBC stage [generated and kindly provided by Dr Laurent Derré, University Hospital Lausanne, Switzerland (16)] and RT4:pT2 (cat. no. HTB-2™), J82:pT3 (cat. no. HTB-1™) and TCC-SUP:pT4 (cat. no. HTB-5™) corresponding to the three stages of disease invasiveness, were purchased from the American Type Culture Collection. Cells were cultured in RPMI-1640 10% heat-inactivated FCS (Gibco; Thermo Scientific, Inc.) at 37˚C in a 5% CO_2 humidified atmosphere. The medium was changed as recommended by the manufacturer and cells were used for the experiment when confluency reached 70-80%.

RNA isolation. Total RNA was extracted from 70 fresh frozen biopsies conserved in RNA Later (Invitrogen; Thermo Fisher Scientific, Inc.) and from four BC cell lines using TRI Reagent (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol. The amount and quality of DNA and RNA were measured by NanoDrop 2000 (Thermo Fisher Scientific, Inc.). The ratio of absorbance at 260 and 280 nm was used to assess purity. A ratio of ~1.8 was accepted as 'pure' for DNA. RNA was considered DNA and protein free if the ratio of readings at 260/280 nm was ~2.

Gene expression study. A total of 1 μg of each of the 70 RNA samples was subjected to reverse transcription independently using the High-capacity cDNA reverse transcription kit according to manufacturer's protocol (Applied Biosystems; Thermo Fisher Scientific, Inc.). cDNAs were subsequently used to perform a SYBR green-based quantitative PCR using the KAPA SYBR FAST Kit (Roche Diagnostics). Enzyme was first activated at 95˚C for 3 min, followed by 40 cycles of denaturation at 95˚C for 3 sec, primer annealing and extension for 20 sec at 60˚C. Samples were amplified in triplicate, and a non-template control was used for each primer pair to control for contamination or primer dimerization. STAT1 and STAT4 levels were normalized to the expression of β2 microglobulin (β2M) used as an internal control gene, using the $2^{-\Delta\Delta Cq}$ formula (17). Primers' sequences are shown in Table II.

Statistical analysis. Statistical analysis of the potential association between clinicopathological features and patients' clinical outcomes was performed using Graph Pad Prism software version 10 (Dotmatics). Unpaired student's t-test was used for the comparison between two groups; for the comparison between multiple groups, two-way ANOVA or the non-parametric (Mann-Whitney or Kruskal-Wallis tests) tests were used. Survival analyses were performed using the Kaplan-Meier method and compared by a log-rank test on Graph Pad Prism software version 10, and the follow-up period was defined from the date of first transurethral resection of bladder tumor treatment to the moment of death for deceased cases, or the date of the last follow up for survivors. $P<0.05$ (two-tailed) was considered to indicate a statistically significant difference.

Results

Differential expression of STAT1 and STAT4 in early vs. aggressive tumors. In the present prospective study, the mRNA level of STAT1 and STAT4 genes were evaluated in both BC cases as well as in BC cell lines mimicking the four state and the four stages of cancer progression. The results are presented

Table I. Clinicopathological characteristics of patients.

Parameter	Total cases	Percentage (%)
Sex		
Male	68	97.14
Female	2	2.86
Age, years		
<50	1	1.43
50-70	44	62.86
>70	25	35.71
Smoking history		
Yes	28	40
No	42	60
Tumor stage		
≤PT1	52	74.29
>PT1	18	25.71
Tumor grade		
Low grade	27	38.57
High grade	43	61.43
Tumor recurrence		
Yes	12	23.08
No	40	76.92
Tumor progression		
Yes	5	9.62
No	47	90.38

Table II. Sequences of primers used in the present study.

Gene name	Primer sequence (5'-3')
β2M	F: GAGGCTATCCAGCGTACTCCA
	R: CGGCAGGCATACTCATCTTTT
STAT1	F: ATCAGGCTCAGTCGGGGAATA
	R: TGGTCTCGTGTTCTCTGTTCT
STAT4	F: TGTTGGCCCAATGGATTGAAA
	R: GGAAACACGACCTAACTGTTCAT

F, forward; R, reverse.

in Fig. 1, where the expression of STAT1 and STAT4 showed no significant association with patient age, sex, or smoking history (Fig. 1A-C).

STAT1 expression was significantly upregulated in patients with muscle-invasive BC (MIBC) compared with those with NMIBC, and in progressive tumors relative to primary and recurrent cases. By contrast, STAT4 expression was slightly higher in NMIBC compared with MIBC cases (Fig. 1D), in low-grade tumors compared with higher-grade stages (Fig. 1E), and in recurrent cases compared with primary and progressive tumors (Fig. 1F), although these differences were not statistically significant.

In BC cell lines, STAT1 and STAT4 patterns are similar to those observed in human primary tissue samples. Indeed, STAT1 expression scored the highest rate in advanced tumor stages, while STAT4 was higher in the NMIBC cell lines and its expression decreased according to tumor invasion (Fig. 1G and H).

STAT1/4 expression as a survival prognostic biomarker in patients with BC. In the present study, the mean and the median of clinical follow-up periods were 44 and 45 months, respectively. Kaplan-Meier analyses were performed to examine the possible correlations between the expression of the studied and patients' clinical outcomes: Disease-free survival (DFS) and overall survival (OS). Based on the median value, patients were stratified into two groups: The low expression group, with mRNA levels below the cohort's median, and the high expression group, with mRNA levels equal to or above the median.

For STAT1, the DFS and OS rates were nearly identical (Fig. 2A and B). The survival curves showed no significant differences between the low expression and the high expression groups (DFS, P=0.450; OS, P=0.396). Interestingly, patients with high STAT4 expression had significantly longer DFS (P=0.005) and OS (P=0.003) than those with low STAT4 expression (Fig. 2C and D).

Correlation between STAT1 and STAT4 expression levels. To explore the potential correlation between STAT1 and STAT4 expression in BC, the expression levels of both genes were compared across patients (Fig. 3). This analysis uncovered a significant positive correlation between the mRNA levels of STAT1 and STAT4 (r=0.691; P<0.0001; 95% confidence interval, 0,5010-0,8185) (Fig. 3A). The positive correlation was sustained in both NMIBC and MIBC subtypes, although STAT1 was upregulated in MIBC and STAT4 in NMIBC. The examination revealed that most patients with high STAT1 expression had also high STAT4 expression (Fig. 3B).

Discussion

Worldwide, significant efforts have been made to uncover the molecular mechanisms and pathogenesis of cancer development and progression. However, the detailed process remains one of the most unsolved aspects of cancer biology (18). Understanding the molecular events occurring at different stages of tumorigenesis can pave the way for the discovery of new biomarkers and improve predictions of disease progression and patient prognosis factors, as demonstrated by recent studies on BC (19-22).

In the present study, STAT1 and STAT4 were investigated as potential biomarkers in BC, building on the concept that transcription factors can serve as critical prognostic indicators in various malignancies. For example, transcription factors such as EGFR (23) and STAT3 in non-small cell lung cancer (9,24) and TWIST1/Vimentin have proven also their utility in detecting urothelial carcinoma of the bladder due to their involvement in key processes such as epithelial-to-mesenchymal transition and metastasis. Similarly, E2F family members (E2F3/5/8) have shown promise as prognostic markers in BC, emphasizing the relevance of transcription factors in tumor biology and their therapeutic potential (25).

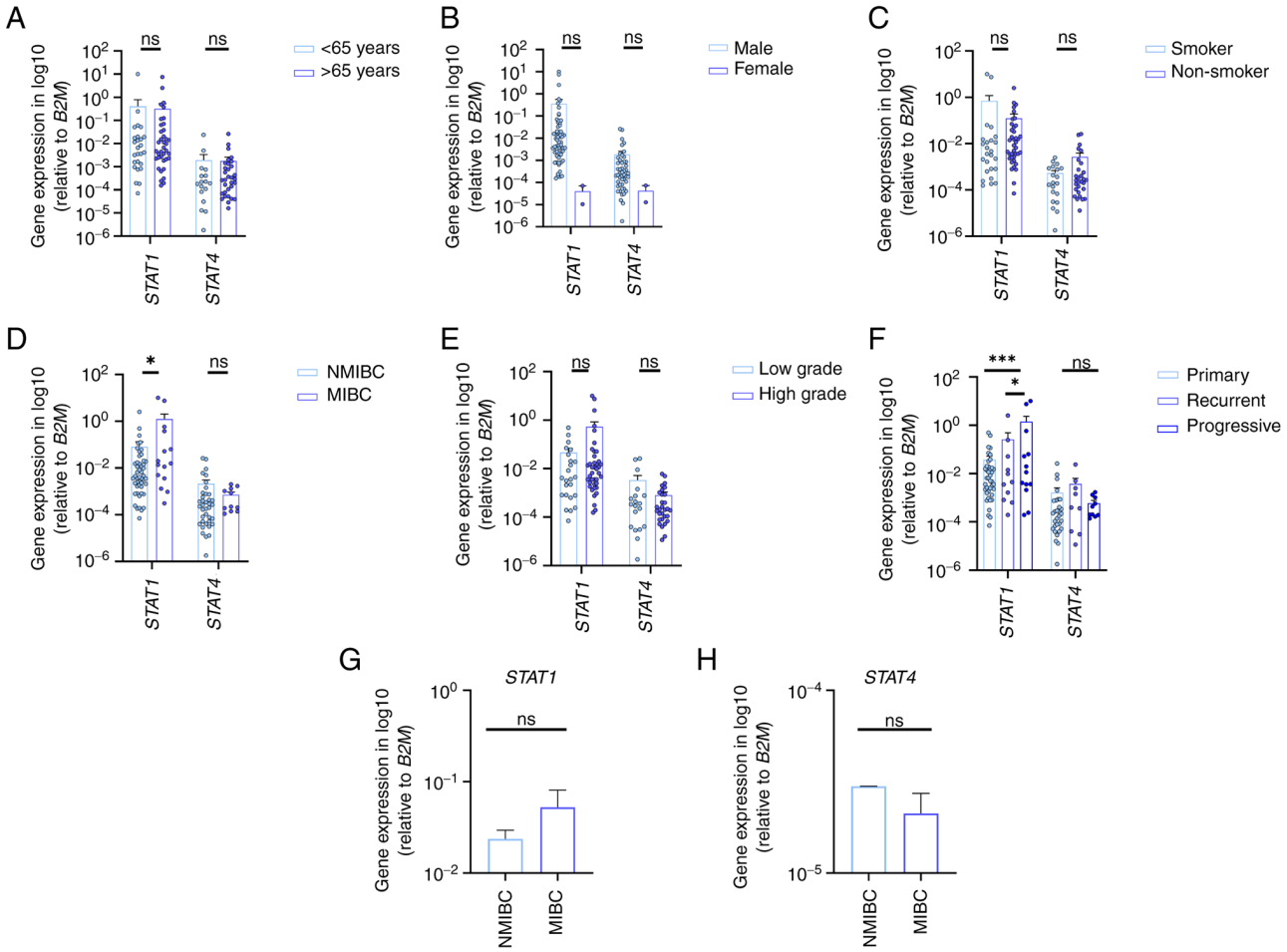

Figure 1. Differential expression of STAT1 and STAT4 in patients with BC and BC cell lines. (A-F) The expression level of STAT1 and STAT4 according to (A-C) patients' demographic data and (D-F) pathological data. (G and H) STAT1 and STAT4 expression in bladder cancer cell lines. *P<0.05 and ***P<0.001. BC, bladder cancer; MIBC, muscle-invasive BC; NMIBC, non-MIBC; ns, not significant.

In the present study, a cohort of 70 samples of BC obtained from patients receiving the same standard of care were used for gene expression investigation and survival analyses. Through the extensive follow-up data that was collected over four years, it was possible to highlight the opposite roles played by STAT1 and STAT4 in bladder tumorigenesis. It was identified that STAT1 is significantly upregulated in advanced stages of BC, in recurrent and in progressing tumors, emphasizing its role as a biomarker of aggressiveness and progression. On the other hand, STAT4 appeared to act as a protective factor in our cohort, with slightly higher expression observed in early-stage cancer biopsies, though not reaching statistical significance.

Furthermore, STAT1 was almost 10-fold overexpressed in the high-grade group. These observations align with a previous study that has shown significantly higher STAT1 expression across a panel of 12 cancer types, with elevated STAT1 levels linked to higher tumor grades in bladder carcinoma (26).

The protective role of STAT4 is experimentally demonstrated by the high level of mRNA detected in non-invasive tumor stages and non-relapsing patients over the 4-year follow-up period. STAT4 has been suggested to induce inflammation and autoimmune diseases, inhibit tumor growth, or promote tumors via regulating numerous facets of the innate and adaptive immune responses (27). According to a previous study, STAT1 and STAT4 tend to increase simultaneously in several immune disorders, including inflammatory bowel disease (15). The present study points to contrasting roles for these two genes and, if validated in larger patient cohorts, they could be utilized as prognostic markers. STAT1 overexpression might indicate aggressive tumors, while high STAT4 expression could be associated with favorable clinical outcomes. Our survival analysis revealed that STAT1 expression was not significantly associated with OS or DFS, as shown in Fig. 2. This unexpected result may reflect several factors: The heterogeneous nature of BC, the influence of other molecular pathways compensating for STAT1 activity, or the sample size limitations of our cohort. It is also possible that STAT1's role in tumor progression depends on context-specific factors, such as interactions with the tumor microenvironment or post-transcriptional modifications affecting its activity. These findings warrant further validation in larger cohorts and functional studies to elucidate the interplay between STAT1, STAT4 and the broader immune landscape in BC.

On the other side, the survival analysis revealed a significantly superior DFS and OS in patients displaying higher STAT4 expression. This improved outcome may be attributed to the crucial role of STAT4 in IFN-γ induction, which is essential

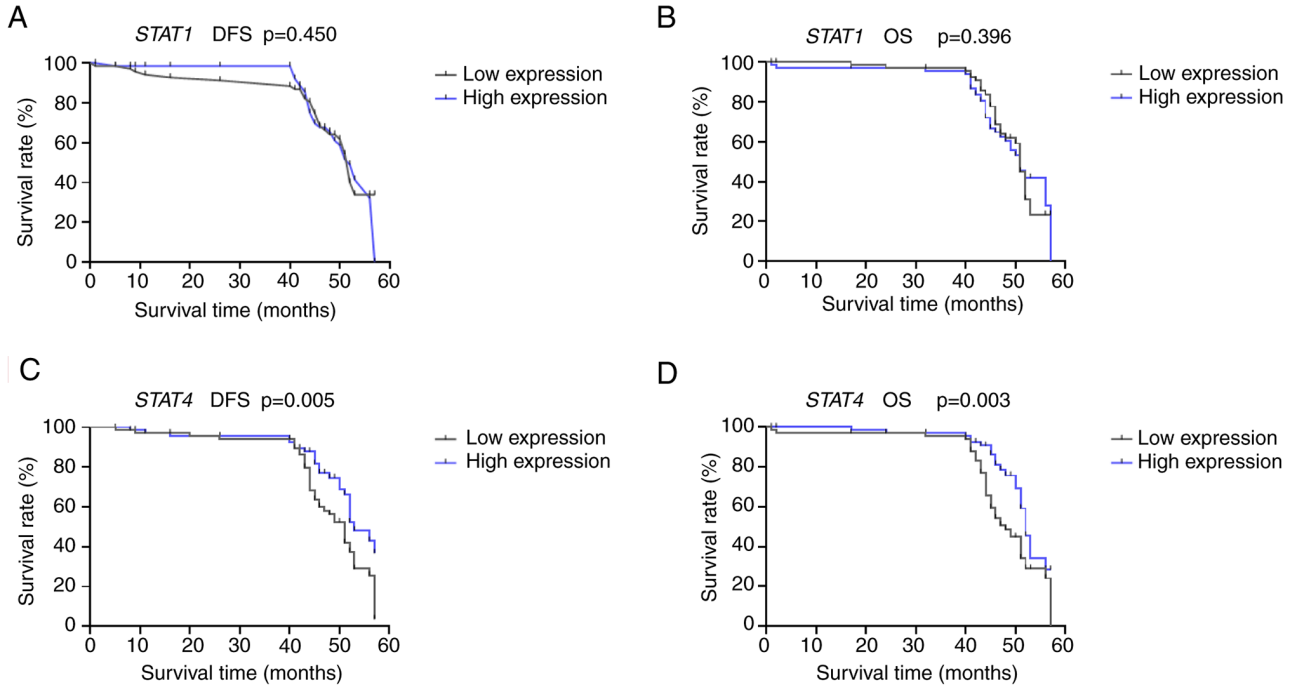

Figure 2. Survival analysis in patients with BC. (A-D) Kaplan-Meier analyses of DFS and OS in patients with BC stratified according to (A and B) STAT1 and (C and D) STAT4 gene expression in patients' samples. Low expression was identified as mRNA level below the median of the cohort, and high expression was identified as mRNA level at or above the median for the cohort. DFS, disease-free survival; OS, overall survival; BC, bladder cancer.

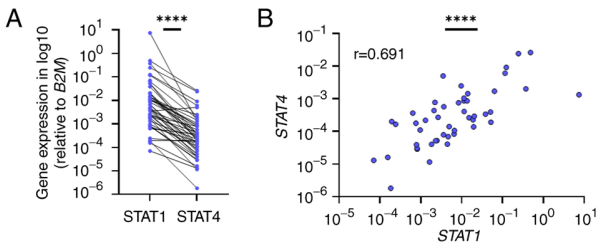

Figure 3. Correlations of gene expression of STAT1 and STAT4. (A and B) Spearman correlation of STAT1 and STAT4 gene expression in patients' samples. ****P<0.0001.

for boosting antitumor immunity. Supporting this idea, STAT4 inhibition has been demonstrated to promote tumorigenesis and predict poor outcomes in cutaneous T-cell lymphoma, hepatocellular carcinoma and gastric cancer (10,11,28-30).

The potential link between the expression profiles of the two genes studied was further investigated. Interestingly, a positive correlation was found between STAT1 and STAT4 expression, indicating that patients with higher STAT1 expression also tend to exhibit higher STAT4 levels. Although STAT1 and STAT4 may have opposing roles in BC, this association suggests a functional relationship likely due to co-regulation from a shared genetic locus. This co-regulation may enable STAT1 and STAT4 to complement each other, working synergistically to modulate antitumor immune responses and revealing an interaction that has not been previously well-defined (15). A similar expression trend was observed in bladder cell lines, where high STAT1 expression was detected in cells from advanced tumor stages, while low STAT1 expression was associated with BU68.08 (representing the NMIBC stage). By contrast, STAT4 expression exhibited an opposite pattern, with lower levels in advanced-stage cells and higher levels in non-invasive BC cells.

To the best of our knowledge, the present study is the first to report differential expression of STAT1 and STAT4 in human BC, using both primary samples and cell lines. These findings indicate that STAT gene expression could be a valuable target for developing new therapies to improve BC management. However, due to the limited sample size and the complex role of STAT signaling in cancer progression, further research is essential to confirm STAT1 and STAT4 as reliable prognostic biomarkers. Exploring the distinct roles of STAT1 and STAT4 in tumor progression and the tumor microenvironment presents a promising avenue for future research. A priority will be to perform targeted knockout and overexpression experiments for STAT1 and STAT4 in BC cell lines. Complementing these *in vitro* studies with *in vivo* experiments using animal models will provide critical insights into how these genes influence tumor growth, metastasis and interaction with the tumor microenvironment in a physiological context. These combined approaches aim to elucidate the detailed mechanisms underlying their roles in cell proliferation, migration and invasion, thereby advancing our understanding of their functional significance and therapeutic potential in BC.

In conclusion, the present study demonstrated that STAT1 expression is significantly upregulated in patients with advanced MIBC and progressing bladder tumors. By contrast, STAT4 expression was higher in NMIBC, although the difference was not statistically significant. In BC cell lines, the expression trends of STAT1 and STAT4 mirrored those observed in human primary tissues. Notably, patients with high STAT4 expression exhibited significantly improved DFS and OS compared with those with low expression, likely due to the enhanced immune response, particularly through the

activation of Th1 cells that aid in controlling tumor growth and recurrence. Additionally, STAT4 influences pro-inflammatory pathways, creating an antitumor environment that limits tumor progression and ultimately improves patient prognosis. As a consequence, the current findings suggested STAT1 and STAT4 as promising biomarkers for prognostic prediction in BC.

Furthermore, it is anticipated that the present study would be a catalyst for collaborative research that could integrate the present findings into clinical trials and potentially develop STAT1 and STAT4-based diagnostic and prognostic tools.

Acknowledgements

Not applicable.

Funding

The present study was supported by a Consolidation grant (grant no. COG-2023-37) from the HES-SO Leading House for the Middle East and North Africa (to EAH).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

EAH designed the study, performed the experiments, analyzed the data, wrote the manuscript, and provided final approval for publication. EAM performed the experiments and analyzed the data. AM analyzed the data and contributed to manuscript writing. HAC, HI and CI performed biostatistical analysis. TM, AA, ABA and OM provided patient samples and follow-up. AA, AM and BL provided intellectual contributions and revised the manuscript. JC and AM provided intellectual contributions, critically revised the manuscript, and provided final approval for publication. EAH and AM confirm the authenticity of the raw data.

Ethics approval and consent to participate

The study protocol was approved (approval no. Ref 82/19) by the Ethics Committee of Biomedical Research from the Faculty of Medicine and Pharmacy of Rabat (Rabat, Morocco). Written informed consent was obtained from each recruited patient prior to sample collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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