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Retrospective Study

Marker Ki-67 is a potential biomarker for the diagnosis and prognosis of prostate cancer based on two cohorts

Song Z *et al*. MKI67 is a biomarker for PCa

Abstract

BACKGROUND

Prostate cancer (PCa) is a widespread malignancy, predominantly affecting elderly males, and current methods for diagnosis and treatment of this disease continue to fall short. The marker Ki-67 (MKI67) has been previously demonstrated to correlate with the proliferation and metastasis of various cancer cells, including those of PCa. Hence, verifying the association between MKI67 and the diagnosis and prognosis of PCa, using bioinformatics databases and clinical data analysis, carries significant clinical implications.

AIM

To explore the diagnostic and prognostic efficacy of antigens identified by MKI67 expression in PCa.

METHODS

For cohort 1, the efficacy of MKI67 diagnosis was evaluated using data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases. For cohort 2, the diagnostic and prognostic power of MKI67 expression was further validated using data from 271 patients with clinical PCa.

RESULTS

In cohort 1, MKI67 expression was correlated with prostate-specific antigen (PSA), Gleason Score, T stage, and N stage. The receiver operating characteristic (ROC) curve showed a strong diagnostic ability, and the Kaplan-Meier method demonstrated that MKI67 expression was negatively associated with the progression-free interval (PFI). The time-ROC curve displayed a weak prognostic capability for MKI67 expression in PCa. In cohort 2, MKI67 expression was significantly related to the Gleason Score, T stage, and N stage; however, it was negatively associated with the PFI. The time-ROC curve revealed the stronger prognostic capability of MKI67 in patients with PCa.

Multivariate COX regression analysis was performed to select risk factors, including PSA level, N stage, and MKI67 expression. A nomogram was established to predict the 3-year PFI.

CONCLUSION

MKI67 expression was positively associated with the Gleason Score, T stage, and N stage and showed a strong diagnostic and prognostic ability in PCa.

Key Words: Marker Ki-67; Prostate cancer; Biomarker; Diagnosis; Prognosis

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Core Tip: Marker Ki-67 (MKI67) has been established to correlate with the proliferation and metastasis of various malignant tumor cells, including those implicated in prostate cancer (PCa). Our objective is to validate the connection between MKI67 and the diagnosis as well as prognosis of PCa, by deploying two distinct patient cohorts from bioinformatics and clinical data. Within the bioinformatics data cohort, comprising 496 PCa tissue samples juxtaposed with 152 normal controls, we ascertained that MKI67 possesses a strong diagnostic ability for PCa along with a moderate prognostic prediction potential. Similarly, through our retrospective analysis of clinical data from 271 PCa patients, we confirmed the potent diagnostic capacity of MKI67 for PCa and its capability to predict prognosis to a certain extent.

INTRODUCTION

Prostate cancer (PCa) is the second most common malignancy worldwide[1] and has become the most prevalent diagnosed carcinoma in American males, accounting for 20% of new diagnoses[2,3]. Although PCa has the highest probability of survival, its

protopathic mortality rate is approximately 10%[2]. In China, PCa has surpassed bladder and kidney cancers in terms of incidence and mortality, and is currently the most common tumor in adult urology[4]. Clinicians must correctly and appropriately diagnose and treat all types of PCa. Prostate-specific antigen (PSA) testing, prostate biopsy, and pathological diagnoses are frequently used to detect and diagnose PCa. However, given the insidious onset of PCa, early symptoms are not obvious, and the disease progresses slowly. Therefore, there is a persistent need to further improve the detection rate to achieve early diagnosis and early treatment and increase the survival rate of patients with PCa. Regarding treatment, androgen deprivation therapy in conjunction with radiotherapy is preferable to other treatments in patients with high-risk PCa[5]. Radical prostatectomy (RP) is one of the most efficacious treatments[6]. However, biochemical recurrence still occurs in approximately 15%-35% of patients after radical surgery[7]. Approximately 29% of these patients eventually experience the recurrence of clinical lesions[8]. Therefore, it is imperative to discover and validate biomarkers that can assist in disease diagnosis and prognosis, thereby aiding clinicians in improving treatment choices.

It is worth noting that all prognostic parameters commonly used in clinical practice for PCa have major limitations. For example, there exists substantial interobserver variation in the Gleason grade, even among specialized urogenital pathologists[9]. Therefore, there is a need for additional prognostic parameters that are not necessarily statistically independent of established parameters but are more reproducible and reliable than established parameters. Despite the recent advances in molecular pathology, immunohistochemistry (IHC)-based biomarkers offer substantial advantages in terms of universal, rapid, and reliable transfer to clinical routine owing to the widespread use of IHC. Increased cell proliferation is a characteristic of cancer[10]. The marker Ki-67 (MKI67) is employed to indicate the proliferation of tumor cells, including the prostate, and is closely associated with epithelial-mesenchymal transition[11]. Typically, the expression of this functionally unknown DNA-binding protein is determined using IHC[12]. Recently, several researchers have found that the

proliferation of MKI67 is related to PCa prognosis[13-15]. Herein, we further evaluated the value of MKI67 expression in the diagnosis and prognosis of PCa by undertaking a comparative analysis of two cohorts, *i.e.*, a bioinformatics database, and clinical data, and attempted to construct a nomogram to guide the assessment of the clinical prognosis of PCa.

MATERIALS AND METHODS

Cohort 1: RNA-sequencing data and bioinformatics analysis

The RNA-sequencing data in transcripts per million (TPM) format containing 496 PCa tissues and 152 normal samples from the database ID named "TCGA_GTEx-PRAD" from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases were uniformly processed by the Toil process from the University of California Santa Cruz (UCSC) Xena (https://xenabrowser.net/datapages/) when first downloaded[16]. Subsequently, the RNA-sequencing data and clinical information of 499 cases of PCa projects accompanied by 52 counterpart samples from database ID "TCGA-PRAD" were recorded from the TCGA database (https://portal.gdc.cancer.gov/). All data formats were converted from level 3 HTSeq fragments per kilobase per million into TPM for further analysis. All the procedures were performed in accordance with the Declaration of Helsinki (as revised in 2013).

Cohort 2: Clinical data

Inclusion and exclusion criteria: Patients with PCa who underwent RP and bilateral pelvic lymph node dissection at the First Affiliated Hospital of Soochow University between January 2018 and January 2019 were analyzed. The inclusion criteria were as follows: (1) Patients with PCa at the first diagnosis; (2) Patients who had not undergone preoperative endocrine therapy, neoadjuvant radiotherapy, or chemotherapy; (3) PCa confirmed by pathology and IHC after PR in all patients; (4) PSA < 0.2 ng/mL at 6-wk postoperative recheck; and (5) Complete clinicopathological features and follow-up data were available. Exclusion criteria were as follows: (1) Combined autoimmune

diseases; (2) Combined other tumors; and (3) Patients or family members who refused to participate in the clinical study. The study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (No. 119, 2021), and all the patients signed informed consent forms before study participation.

IHC: Prostate tissue specimens were collected and fixed in a 10% formaldehyde solution. The tissue samples were embedded in paraffin and sliced for immunohistochemical analysis. Paraffin slices were subsequently subjected to sequential dewaxing, hydration, washing with phosphate-buffered saline (PBS), and rinsing with citric acid buffer. To inactivate the endogenous peroxidase, a 3% hydrogen peroxide solution was applied and allowed to react for 10 min at room temperature. The slices were then washed again with PBS again before the addition of 50 μL of 5% bovine serum albumin, which was incubated at room temperature for 10 min. After adding the primary antibody against MKI67 (1:200 dilution), the slices were incubated overnight at 4 °C and washed with PBS. Following the addition of the secondary antibody, the slices were incubated at room temperature for 60 min and washed again with PBS. The sections were then treated with the DAB color reagent for 5 min, re-stained with hematoxylin, dehydrated using a graded ethanol series, and sealed with neutral resin for microscopic analysis.

Criteria for determining results: Given that MKI67 is primarily expressed in the nucleus, the appearance of pale yellow, brown-yellow, or brown granules in the nuclei at 200-fold magnification was considered positive. For a section to be judged positive, ten microscopic views at 400-fold magnification were randomly observed and scored according to the proportion of positive cells and staining intensity[17].

General information: The clinical data for patients with PCa included age, body mass index (BMI), diabetes and hypertension, preoperative PSA level, postoperative Gleason score, IHC findings, pathological staging, and follow-up time, with a follow-up cutoff

date of December 31, 2021. The endpoint event was progression-free interval (PFI), which was defined as a decrease in PSA level to < 0.2 ng/mL in patients six weeks after RP, and a PSA ≥ 0.2 ng/mL was monitored during the follow-up. Herein, we included 271 patients with an average age of 69.50 ± 6.74 years, mean BMI of 23.94 ± 3.22 kg/m^2, and mean preoperative PSA of 19.15 ± 16.81 ng/mL. MKI67 expression ranged from 1% to 95%, with a mean of 9.38% ± 11.87%, and biochemical recurrence was observed in 37 cases. The duration of follow-up varied from 5 to 44 mo, with an average of 34.83 ± 8.30 mo. Overall, 141 patients with hypertension and 40 with diabetes mellitus were enrolled.

Sample size calculation: Calculations using R software and verification by statisticians from the First Affiliated Hospital of Soochow University confirmed that the minimum sample size required for this study was 38. A total of 271 patients were included in this study, providing statistical validity for the conclusions of the study. The statistical code is as follows[18]: "pA = 0.04, pB = 0.26, kappa = 1, alpha = 0.05, beta = 0.20, nB = [pA × (1 - pA)/kappa + pB × (1 - pB)] × [qnorm(1-alpha/2) + qnorm(1-beta)]/(pA-pB)^2, ceiling(nB) # 38, z = (pA-pB)/sqrt[pA × (1 - pA)/nB/kappa + pB × (1-pB)/nB], power = pnorm[z - qnorm(1-alpha/2)] + pnorm[-z - qnorm(1-alpha/2)]".

Statistical analysis

Data analyses were performed using the R software (version 3.6.3; The R Foundation for Statistical Computing, Vienna, Austria). Enumeration data are shown as *n* (%), and measurement data are shown as the mean ± SD. For analysis, patients were divided into two PSA groups (< 4 ng/mL group and ≥ 4 ng/mL group) and six TNM groups (T2 and T3-4, N0 and N1, M0 and M1). Patients were also classified into low/medium-(≤ 7 points) and high-risk (> 7 points) Gleason score groups. Low and high MKI67 expression groups were classified based on the median value of MKI67 expression. Differences between MKI67 expression and clinicopathological features were compared using the Wilcoxon rank-sum test [ggplot2 (version 3.3.3)]. The receiver operating

The expression of MKI67 in prostate tissue is shown in Figure 2. We found that MKI67 expression was significantly and positively associated with Gleason score, T stage, and N stage ($P < 0.05$) but negatively correlated with PFI ($P < 0.001$) (Figures 3A-D). The Kaplan-Meier method showed that the MKI67 high-expression group had a lower PFI survival rate in than the low-expression group (HR = 0.11, $P < 0.001$) (Figure 3E). Moreover, the time-ROC curves showed that MKI67 expression had robust prognostic power in PCa (1 year, AUC = 0.868; 2 years, AUC = 0.857; 3 years, AUC = 0.874) (Figure 3F). Multivariate Cox regression analysis was performed to identify risk factors, including PSA level, N stage, and MKI67 expression ($P < 0.05$) (Table 1). Based on these results, a nomogram for predicting the 3-year PFI was established for clinical use (Figure 4).

DISCUSSION

PCa is well-known to pose a serious threat to the lives and health of males. Although the prevalence of PCa in China is substantially lower than that in Western countries, it is growing annually owing to changes in diet and an aging population. Currently, the commonly used diagnostic approaches for PCa include PSA, prostate puncture biopsy, and pathological diagnosis, with prognosis mostly based on the PSA level, TNM stage, and Gleason score. However, serum PSA levels are markedly limited in the diagnosis of early PCa, which may result in the overdiagnosis or overtreatment of patients and other situations[19]. Prostate biopsy has certain requirements regarding the tumor sampling site and the physician's operating technique, and the potential for misdiagnoses or missed diagnoses cannot be excluded. Furthermore, owing to the heterogeneity of tumors, a single prognostic factor appears to be insufficient to predict patient prognosis. Molecular markers can be used to reduce overtreatment. Despite the widespread interest in different biomarkers and accumulating evidence, especially in the genomic signature era[20], a limited number of biomarkers have survived the test of reproducibility to enter clinical application.

Compared with other prostate biomarkers, MKI67 is an attractive biomarker owing to its universal applicability and reproducibility[21]. MKI67 is widely used in routine practice and is measured in a semi-quantitatively manner with a low failure rate. Moreover, MKI67 is highly expressed in circulating cells but strongly downregulated in resting G0 cells[22], making MKi67 a clinically important marker of cell proliferation for grading several cancers. MKI67 has demonstrated a good prognostic value in breast, lung, and cervical cancers[23-25]. In addition, because the PCa grading system does not consider the cell proliferation rate, detecting the proliferation rate could yield additional prognostic information. Several studies have shown that MKI67 is a promising early diagnostic and prognostic biomarker for PCa[14,26-28]. However, there was substantial heterogeneity among the studies, including different endpoints, cohort types, sample sizes, and cutoff values, as well as a lack of further assessment of MKI67 expression for PCa diagnosis and prognosis. Notably, the results of the current study have several important implications. First, analyzing and validating both cohorts, we found that MKI67 had a high diagnostic value for PCa, with markedly higher expression in PCa tissues than that in normal tissues and positively related to the Gleason score, T stage, and N stage, but not the M stage; these findings are consistent with those reported previously[29]. Collectively, these findings suggest that MKI67 protein expression is associated with aggressiveness and metastasis. Moreover, our results suggest that immunohistochemical techniques can be used to detect the expression of markers to locate the clinical stage of patients, facilitating the development of more appropriate treatment plans to increase the benefits for patients with PCa and improve their survival prognosis. Second, MKI67 expression negatively correlated with PFI in the latest clinical data validation, which is consistent with findings reported previously[27,30-33]. In addition, MKI67 expression was strongly associated with the prediction of 3-year PFI, facilitating the identification of patients with poor prognosis in the clinic and the tailoring of intensive treatment strategies within a reasonable clinical turnaround time. Third, Cox regression analysis identified PSA level, N stage, and MKI67 expression as

risk factors affecting PCa prognosis and established a nomogram to predict the 3-year PFI for clinical application.

Although several independent studies and accumulating evidence persistently propose new biomarkers, MKI67, the most widely studied molecular biomarker in PCa, should be investigated in prospective studies to establish personalized cancer treatment and clinically effective molecular prognosis. In the future, we plan to further elucidate the mechanistic pathways underlying the role of MKI67 in the development of PCa to identify potential targets for the next step in targeted therapy.

Limits of the study

This study had some limitations. First, the sample volume of the clinical cohort in this study was small, including only one case with the M stage, although MKI67 expression did not correlate with the M stage in the bioinformatics analysis. Age, clinical stage, Gleason score, and PSA levels have been found to affect the prognosis of patients with PCa[34]. Age was not associated with patient prognosis in the univariate analysis in this study, whereas PSA level, N stage, and MKI67 expression were related to prognosis in the Cox multifactorial analysis, which may be linked to the small sample size of our study. Meanwhile, the predictive capacity of MKI67 expression on the 3-year PFI rate tended to differ between the two cohorts; therefore, the observed results need to be validated in multicenter trials. Second, the present study showed a positive association between MKI67 expression and well-established prognostic variables, such as PSA, Gleason score, and disease stage; however, our findings were insufficient to precisely quantify the extent of the relationship between MKI67 and outcome. Finally, although we presented a 3-year PFI, the follow-up period was short, and no prospective follow-up was conducted; therefore, other hard endpoints could not be stated, which could have biased the results. Despite limitations, our findings could facilitate further investigations among patients with PCa, both prospectively and as archival materials.

CONCLUSION

By comparatively analyzing bioinformatics databases and clinical data, we found that MKI67 expression was positively correlated with the Gleason score, T stage, and N stage, which would help in establishing the clinical stage of patients and thus develop more appropriate treatment plans. MKI67 is a highly effective diagnostic and prognostic parameter for PCa, and a nomogram for predicting the 3-year PFI was established to facilitate its clinical application. To overcome the limitations of this study, prospective validation of our findings is required to verify their clinical validity.

ARTICLE HIGHLIGHTS

Research background

Prostate cancer (PCa) represents a serious health threat to elderly men as a malignant tumor. Presently, methodologies available for the diagnosis and treatment of PCa are, regrettably, still lacking. Given that marker Ki-67 (MKI67) has been linked with the proliferation and metastasis of PCa cells, it holds substantial clinical meaning to apply both bioinformatics and clinical data to further corroborate the association between MKI67 and the diagnostic and prognostic aspects of PCa.

Research motivation

By establishing the link between MKI67 and PCa, we pave the way for innovative molecular targets and therapeutic approaches for the future diagnosis and treatment of PCa.

Research objectives

To investigate the efficacy of antigen identified by MKI67 expression in the diagnosis and prognosis of PCa.

Research methods

This study undertook a retrospective analysis utilizing both bioinformatics and clinical data. The association between MKI67 expression and various clinicopathological

features was assessed using the Wilcoxon rank-sum test. The diagnostic efficacy of MKI67 expression was conveyed *via* the receiver operating characteristic (ROC) curve. The Kaplan-Meier method was employed to elucidate the progression-free interval (PFI) survival rates in PCa patients. Meanwhile, the time-ROC curve was utilized to predict the 1-, 2-, and 3-year survival rates of the PFI in PCa. Both univariate and multivariate Cox regressions were performed to evaluate the relationship between genetic and clinicopathological characteristics. Lastly, a nomogram was constructed using the rms package.

Research results

In the bioinformatics data, MKI67 expression demonstrated a significant correlation with prostate-specific antigen (PSA), Gleason Score, T stage, and N stage. The ROC curve pointed to a robust diagnostic capacity, while the Kaplan-Meier method indicated that MKI67 expression had a negative correlation with PFI. Moreover, the time-ROC curve exhibited a modest prognostic capability of MKI67 in PCa. In the clinical data, MKI67 expression was significantly tied to the Gleason score, T stage, and N stage, and it was negatively linked to PFI. The time-ROC curve displayed a more substantial prognosis for MKI67 in PCa. A multivariate COX regression analysis was conducted to pinpoint risk factors, which included PSA, N stage, and MKI67 expression. A nomogram was subsequently developed to project 3-year PFI.

Research conclusions

Through comparative analysis of bioinformatics databases and clinical data, MKI67 expression positively correlated with Gleason score and T and N stages, aiding in pinpointing patient's clinical stages for better treatment planning. MKI67 serves as an efficient diagnostic and prognostic tool for PCa, and a nomogram was constructed for predicting 3-year PFI, enhancing its clinical utility.

Research perspectives

In light of the limitations of this study, future prospective validation is necessitated to confirm the clinical relevance of MKI67 in relation to PCa.

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