ORIGINAL RESEARCH

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KIF4A: A potential biomarker for prediction and prognostic of prostate cancer

Abstract

Purpose: To investigate the clinical relevance and biological function of the kinesin super-family protein 4A (KIF4A) expression in prostate cancer (PCa).

Methods: We examined 1) the relationship between the expression of KIF4A and clinico-pathological characteristics of PCa patients using a tissue microarray and the Cancer Genome Atlas database, 2) the prognostic value of KIF4A expression in patients using Kaplan-Meier plots and 3) the functions of KIF4A in LNCaP and DU145 cells, such as cell proliferation, cell cycle and cell apoptosis.

Results: Compared with normal prostate, the mRNA and protein expressions of KIF4A were up-regulated in PCa. The up-regulation expression rates of KIF4A in PCa were significantly related to the Gleason score ($P < 0.001$). The elevated KIF4A level also predicted independently a poor biochemical recurrence-free and disease-free survival of PCa patients. The ectopic expression of KIF4A in PCa was significantly positively associated with the capability of migration and proliferation.

Conclusion: Over-expression of KIF4A correlated with the aggressiveness of PCa and with biochemical recurrence-free survival. Thus, KIF4A is a potential diagnostic indicator and therapeutic target for PCa.

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Introduction

Prostate cancer (PCa), a disease specific to humans, is the second most fatal cancer for men. Accounting for substantial morbidity and mortality, with over 174,650 new patients and 31,620 decedents in 2019, PCa remains worrisome. It has been predicted the number of PCa patients would reach 1.3 million in the United States of America from 2019 to 2030 [1,2]. A few relatively new approaches, including enzalutamide and abiraterone treatments, significantly improve overall survival rate and the progression of PCa-free status [3,4]. However, the options of therapies are considerably limited for those with advanced PCa [5-9]. To further the study the progression of this tumor, identification of effective prognosis biomarkers is required.

The kinesin superfamily proteins (KIFs), also known as microtubule-dependent motor proteins, can move along microtubules and transport cytoplasmic cargos with energy obtained via ATP hydrolysis. The KIFs are classified into 14 subgroups, according to the different function features [10,11]. One of these proteins, KIF4A, has been reported to be involved in various biological activities, such as cell division, chromosome condensation, spindle organization and cytokinesis [12-14]. The abnormal expression of KIF4A in eukaryotic cells is thought to participate in spindle separation, response in DNA damage and

aneuploidy of new cells. Moreover, dysregulation of KIF4A accelerates the progressions of many cancers, including lung cancer [12,15], colorectal cancer [17,18], breast cancer [16,19], oral cancer [20], cervical cancer [21], renal cell carcinoma [22] and hepatocellular carcinoma [23-25], as well as hepatitis B virus-induced liver cancer [26]. Nevertheless, the expression profile and the function of KIF4A in PCa remain largely unknown.

Materials and methods

Patients and tissue samples

A tissue microarray (TMA, n=80), including samples from 73 prostates from primary PCa patients and seven prostates from healthy people, was obtained from Creative Bioarray (Cat No: PRTMA053). (Information on the 73 patients involved in our study is available upon request). Patients treated with chemotherapy or radiotherapy before the surgery, were excluded from this study. In order to quantify the mRNA expression of KIF4A and perform the survival analysis, the clinical information and gene expression data were collected from the Cancer Genome Atlas (TCGA) database, and included 499 prostate cancer tissues (supplementary material is available upon request). The details of clinical features of all patients in this study were showed in Table 1.

TABLE 1. Correlation of KIF4A expression with clinicopathological characteristics in patients with prostate cancer

Clinical features	TMA*				TCGA			
	# Cases	Low, n (%)	High, n (%)	P	# Cases	Low, n (%)	High, n (%)	P
Tissue								
Cancer	73	21(28.8)	52(71.2)	<0.001**	499	249(49.9)	250(50.1)	-
Non-cancer	7	7(100.0)	0(0.0)	-	-	-	-	-
Age (years)								
<60	14	9(64.3)	5(35.7)	0.015*	202	112(55.4)	90(44.6)	0.023*
≥60	66	19(28.8)	47(71.2)	-	296	136(45.9)	160(54.1)	-
Serum PSA levels (ng/ml)								
<4	-	-	-	-	414	217(52.4)	197(45.6)	0.017*
≥4	-	-	-	-	27	8(29.6)	19(70.4)	-
Gleason score								
<7	12	8(66.7)	4(33.3)	<0.001**	44	38(86.4)	6(13.6)	<0.001**
≥7	53	8(15.1)	45(84.9)	-	454	210(46.3)	244(53.7)	-

Pathological grade								
pT2	34	12(35.3)	22(64.7)	0.034*	-	-	-	-
pT3/4	31	4(12.9)	27(87.1)	-	-	-	-	-
Tumor stage								
T1	2	2(100.0)	0(0.0)	0.055	178	111(62.4)	67(37.6)	0.001**
T2	42	14(33.3)	28(66.7)	-	174	80(45.9)	94(54.1)	-
T3	21	3(14.3)	18(85.7)	-	53	19(35.8)	34(64.2)	-
T4	8	2(25)	6(75)	-	2	1(50)	1(50)	-
Lymph nodemetastasis								
N0	53	19(35.8)	34(64.1)	0.025*	345	173(50.1)	172(49.9)	0.003*
N1	20	2(10.0)	18(90.0)	-	80	26(32.5)	54(67.5)	-
Distant metastasis								
M0	50	14(28.0)	36(72.0)	0.520	456	224(49.1)	232(50.9)	0.515
M1	23	7(30.4)	16(69.6)	-	3	1(33.3)	2(66.7)	-

* low = ≤ 4 ; high = >4

Abbreviations: PSA, prostate specific antigen; TCGA, Cancer Genome Atlas; TMA, tissue microarray

Immunohistochemistry analysis

The paraffin-embedded prostate tissues were collected and deparaffinized. The primary antibody anti-KIF4A (rabbit polyclonal antibody, ab122227, Abcam, UK) was used as a dilution of 1: 600, at 4°C, according to the previous studies [27].

Cell lines and cell culture

The human PCa cell lines LNCaP, Du145, PC3, 22RV1 and BPH-1 were obtained from the American Type Culture Collection (ATCC, USA) and RWPE-1 was a gift from Guangzhou Hui Yuan, Yuan Biomedical Technology Limited, China. LNCaP, Du145, PC3, 22RV1 and BPH-1 were maintained in RPMI 1640 medium, and RWPE-1 was cultured in keratinocyte-SFM. All cell lines were cultured as described in a previous study [27].

Plasmids and lentiviral transfection

Overexpression of KIF4A in PCa cells were established as described previously [27].

CCK8 cancer cell growth assay

Cell growth was measured using a reagent from Beyotime (Cat No. C0039; Beyotime Biotechnology, China). Triplicate samples of $5 \times 10^4/2$ mL suspension cells were inoculated into 96-well plates. After culturing for 4 h, 24 h, 48 h and 72 h, the number of cells was measured by Cell Proliferation Reagent CCK-8 according to the manufacturer's instructions.

Analyses of apoptosis and cell cycle

DU145 and LNCaP cells were determined using an Annexin V-APC/ 7AAD kit (Cat No. 4224750, Multisciences, China) and propidium iodide staining (Cat No. ST512, Beyotime Biotechnology) according to the reagents' protocols.

Cell transwell and scraping migration assays

The DU145 and LNCaP cell migrations were detected by scraping and transwell migration assays as described previously [28].

FIGURE 1. Immunohistochemical staining for KIF4A in PCa and normal prostate tissues in our TMA samples

(A) Full view of the immunohistochemistry staining for KIF4A: immunostaining occurred strongly in the cytoplasm in cancer cells of PCa tissues, but at intermediate levels in normal prostate tissues.

(B) Expression level of KIF4A in PCa tissues was significantly higher than in normal prostate tissues (IRS: cancer=4.78±1.42 vs. normal=2.14±1.07, $P=0.000008$; Gleason score <7=4.17±1.19 vs. Gleason score ≥7=5.16±0.91, $P=0.002$).

(C-F) Immunostaining of KIF4A high, medium and low expression. Immunohistochemical staining of KIF4A occurred strongly in the cytoplasm of cancer cells, but only moderately in normal prostate tissue. The percentage of high expression and low expression KIF4A staining in the different groups by the level of Gleason score (=2+4, =2+2, =2+1 and normal tissue).

RNA extraction and quantitative real-time PCR

Expression levels of KIF4A mRNA in PCa cell lines such as LNCaP, Du145, PC3, 22RV1, BPH-1 were determined by qRT-PCR according the manufacturer's instructions. The sequences of primer are as follows: β -actin, F: TGGCACCAGCACAAATGAA; R: CTAAGTCATAGTCCGCCTAGAAGC; KIF4A, F: CGCCGCCGCAAACGTC, R: TGCCTCTTACGGTCTCGTTCTT.

Western blot analysis

Expression levels of KIF4A proteins in PCa cell lines, LNCaP and DU145 were detected by western blot analysis as described previously [28]. Antibodies GAPDH (1:1000, BM0627) and KIF4A (1:1000, ab122227) were used.

Statistical analysis

Statistical analyzes were performed using SPSS 22.0 software (SPSS, USA). Continuous variables were expressed as mean ± SD. Pearson's chi-squared tests and Fisher's exact test were used to analyze the association of KIF4A mRNA expression with clinico-pathological characteristics. Student's T-tests were used to analyze the association of KIF4A protein expression with clinico-pathological characteristics. Overall survivals were analyzed using Kaplan-Meier method. Univariate analysis comparisons and multivariate survival comparisons were performed using Cox proportional hazard regression models. The TCGA and Genotype-Tissue Expression (GTEx) data were acquired from the Gene Expression Profiling Interactive Analysis (GEPIA) for differential expression analysis on Box Plots. Differences were statistically significant when $P<0.05$.

Results

Expression of KIF4A in PCa tissue

To study the effect of KIF4A in prostates of PCa patients, we first measured the proteins of KIF4A in PCa and healthy prostates via immunohistochemical analysis. The KIF4A protein expression levels were determined as low (immunoreactivity score (IRS): 0-4) and high (IRS: 4-12). As shown in Figure 1, KIF4A protein expression were detected at high levels in the cytoplasm and nucleus in PCa tissues, while they occurred at intermediate levels in non-PCa tissues. Among the 73 PCa samples, 21 (28.8%) were classified as low groups and 52 (71.2%) of them were classified as high groups. The expression of KIF4A proteins in PCa tissues were markedly upregulated, compared with that of non-PCa tissue ($P<0.01$, Figure 1B). Notably, the immunostaining of the KIF4A proteins in samples with high Gleason scores (≥7 ng/ml) was more

FIGURE 2. KIF4A serves as an independent prognostic factor

(A) High KIF4A expression is associated with poorer disease-free survival of PCa patients ($P=0.000$, log-rank test). (B) High KIF4A expression relevant to poorer BCR free survival of PCa patients ($P=0.000$, log-rank test)

evident than those with low Gleason scores (<7 ng/ml) ($P=0.02$, Figure 1B).

Expression of KIF4A is associated with clinico-pathological characteristics

The correlation of KIF4A expressions with different clinico-pathological features of PCa patients is summarized in Table 1. Given the high groups correspond to high Gleason scores ($P<0.001$) in TMA, we found that the IRS was significantly positively associated with the Gleason score. We analyzed 499 PCa patients from TCGA database to investigate the relationship between KIF4A expression and clinico-pathological characteristics. As shown in Table 1, 49.9% ($n=249/499$) of the samples were classified as low group and 50.1% ($n=250/499$) were classified as high group. The upregulation of KIF4A protein expression was positively correlated with clinico-pathological characteristics.

Elevated KIF4A levels as a prognostic factor of PCa

We examined whether KIF4A expression was associated with biochemical recurrence-free (BCR-free) survival, overall survival and disease-free survival for PCa patients samples from TCGA database by Kaplan-Meier analysis. High KIF4A expression (high group) in PCa patients appears to lead to lower levels of BCR-free survival and

disease-free survival and low KIF4A expression (low group) in PCa patients lead to higher levels of BCR-free survival and disease-free survival. These data suggest that KIF4A is more likely to be detected in PCa-caused lesions ($P=0.000$, $P=0.000$, respectively, Figure 2). No difference was observed in overall survival ($P=0.058$).

To further determine whether KIF4A expression could be an independent prognostic factor for PCa, the prognostic value of KIF4A expression in PCa patients was assessed using univariate and multivariate Cox regression models. Univariate analysis revealed that KIF4A expression ($P<0.001$), prostate specific antigen (PSA) ($P<0.001$), tumor invasion ($P<0.001$) and lymph node stage ($P<0.034$) were found to be significant prognostic factors for the BCR-free survival in PCa patients (Table 2).

Multivariate analysis confirmed that high KIF4A expression remained an independent significant predictor of BCR-free survival ($P=0.02$, HR=2.133, 95% CI=1.127-4.038) in PCa patients (Table 2).

TABLE 2. Prognostic value of KIF4A expression for the BCR free survival by Cox proportional hazards model

Variable	BCR free survival	
	HR (95%CI)	P
Univariate analysis		
Age (<60 or ≥ 60) (years)	1.319(0.773-2.250)	0.310
Gleason score(<7 or ≥ 7)	3.033(0.740-12.423)	0.123
Tumor invasion (T1,T2,T3 or T4)	2.726(1.856-4.002)	$<0.001^{**}$
Lymph Node Stage (N1 or N0)	1.879(1.049-3.365)	0.034*
Distant metastasis (M1 or M0)	3.536(0.488-25.641)	0.212
PSA (<4 or ≥ 4)	10.426(5.309-20.474)	$<0.001^{**}$
KIF4A expression(low or high)	2.987(1.639-5.443)	$<0.001^{**}$
Multivariate analysis		
Tumor invasion (T1,T2,T3 or T4)	2.66(1.737-4.074)	$<0.001^{**}$
PSA (<4 or ≥ 4)	6.697(3.238-13.853)	$<0.001^{**}$
KIF4A expression (low or high)	1.941(1.035-3.642)	0.039*

FIGURE 3. Overexpression of KIF4A accelerated proliferation, cell cycle and migration progression of PCa cells, but has no effect on apoptosis cells.

(A) RT-qPCR analysis of KIF4A expression in PCa cells. (B) and (C) RT-qPCR and WB analysis of KIF4A expression in DU145 cells and LNCaP cells. (D) and (E) Cell proliferation in DU145 and LNCaP cells. (F) Cell apoptosis assays. (G) Cell cycle assays. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus vector.

Expression of KIF4A is associated with cell proliferation, cell cycle progression and migration in prostatic cells

To gain insight into functional role of KIF4A in prostatic cells, we explored the mRNA expression of KIF4A in diverse prostatic cells (RWPE-1, BPH-1, 22RV1, LNCaP, DU145 and PC3). As shown in Figure 3A, the relative expression of KIF4A was significantly down-regulated in 22RV1, LNCaP and DU145 cells, when comparing with that in the BPH-1. Therefore, DU145 (androgen-independent) and LNCaP (androgen-dependent) cells, both of which are PCa cells, were selected as target cells for upregulation experiments. The relative KIF4A mRNA expression levels in DU145 and LNCaP cells were tested by quantification polymerase chain reaction (qPCR) and the protein levels of KIF4A in these cells were detected via western blot, showing the overexpression of KIF4A was successfully established (Figure 3B and 3C). Then, we performed CCK-8 assays to assess cell viability at different points in time. As shown, the overexpression of KIF4A resulted in an increase in cell viability in DU145 and LNCaP cells, which also suggested that KIF4A could affect proliferation of these cells (Figure 3D).

FIGURE 4. Overexpression of KIF4A impaired migration.

(B)

(C)

(A) Transwell migration assays. (B) and (C) KIF4A overexpression increases the cell migration rate in wound-healing assays in LNCaP (B) and in DU145 (C).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus vector.

To evaluate whether KIF4A was significantly positively associated with the proliferation of PCa cells, we determined apoptosis and cell cycle through flow cytometric analysis. The results revealed that the distribution of cells in S (DNA synthesis) and G2 (cell growth) phases was markedly enhanced after KIF4A overexpression but did not impact cell apoptosis (Figure 3E). Finally, wound-healing and transwell assays were measured to confirm cell motility, presenting KIF4A overexpression stimulated the capacity of wound closure and increased the cell migration (Figure 4A-C). In short, these findings suggest that KIF4A may play a crucial role in PCa proliferation and migration.

Aberrant expression of KIF4A in multiple human cancer types

The pan-cancers analysis was performed using GEPIA online tool. The result showed that the KIF4A was up-regulated in adrenocortical carcinoma, bladder urothelial carcinoma, breast invasive carcinoma, cervical squamous cell carcinoma and endocervical adenocarcinoma, colon adenocarcinoma, lymphoid neoplasm diffuse large B-cell lymphoma, esophageal carcinoma, glioblastoma multiforme, head and neck squamous cell carcinoma, liver hepatocellular carcinoma, lung adenocarcinoma, lung

squamous cell carcinoma, ovarian cystadenocarcinoma, pancreatic adenocarcinoma, rectum adenocarcinoma, skin cutaneous melanoma, stomach adenocarcinoma, testicular germ cell tumors, thymoma, uterine corpus endometrial carcinoma and uterine carcinosarcoma compared with adjacent normal tissues, while it was down-regulated in acute myeloid leukemia. Representative data for adrenocortical carcinoma, bladder urothelial carcinoma, breast invasive carcinoma, cervical squamous cell carcinoma and endocervical adenocarcinoma are shown in Figure 5 (full set of figures for pan-cancer dataset available upon request). These findings, combined with our results for PCa, suggest that KIF4A is likely to be considered a general oncogene.

Discussion

The KIF motor proteins, which include 14 family members, are of great importance for cellular biological processes such as spindle reorganization, chromosome compaction and constraints of excitatory synaptic transmission among normal cells [11-14]. The dysregulation of KIF4A expression and function could lead to tumorigenesis because of the role of KIF4A in these processes. Accumulated evidence has demonstrated that abnormal

FIGURE 5. Aberrant expression of KIF4A in multiple cancer types.

For each figure, the area marked in red represents the tumor group, while the area marked in grey represents normal group.

Abbreviations: ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma. * $P < 0.01$.

KIF4A expression is involved in multiple human cancer, including hepatocellular carcinoma, glioblastoma, oral cancer, lung cancer, breast cancer, colorectal cancer and clear cell renal cell carcinoma [29-33]. Consistent with previous observations, enhanced expression of KIF4A has been observed in 71% of PCa patients compared with healthy samples [36]. KIF4A staining in both the cytoplasm and nucleus was also described in gastric carcinoma as well as oral cancer [20,37]. These findings of higher KIF4A expression in PCa prostate versus normal tissue support the hypothesis that KIF4A may serve as a biomarker of neoplasia. Unfortunately, the TMA did not contain enough PCa patient samples, especially those in T1 (tumor stage). Further studies with more samples would be required to evaluate this hypothesis. The up-regulation of KIF4A expression has been observed in a large cohort of patients with different tumors [32].

The KIF4A expression was associated with the clinicopathologic parameters of PCa patients and Gleason scores [31,36], which indicates that the up-regulation of KIF4A is essential for the progression of this cancer. The clinically prognostic value of KIF4A expression was validated in breast cancer [38], bladder cancer [39] and renal clear cell carcinoma [40]. Evaluated via Kaplan-Meier plots and Cox regression analysis, the prognostic value of KIF4A up-regulation showed an association with shorter BCR-free survival and disease-free survival.

The correlation of higher levels of KIF4A mRNA with shorter overall survival in divergent tumor has also been reported [32]. In addition, the KIF4A expression and PSA were shown to be independent predictors of BCR-free survival by multivariate analysis. Consequently, the expression of KIF4A could be used as a biomarker to recognize PCa patients with a high risk of tumor recurrence.

In addition, we used GEPIA online tool to analyze KIF4A expressions in other malignancies, and found that the relative KIF4A mRNA levels were higher in specific tumors, compared with normal tissue. Recent studies have demonstrated that abnormal KIF4A expression is involved in the progressions of multiple human cancers [15,17,18,20,32,33]. These results support the suggestion that KIF4A may serve as a general oncogene.

Uncontrollable proliferation is a hallmark of malignant tumors. A large body of evidence has demonstrated that KIF4A expression is upregulated in several cancers. Minakawa et al. found that a large percentage of oral squamous cell carcinoma cells were arrested in G2/M phase by spindle assembly checkpoint activation with KIF4A depletion [20]. Huang et al. reported that overexpression of

KIF4A in hepatocytes may contribute to uncontrolled chromosome segregation and promote cell proliferation through activation of Akt signaling [25]. Cao et al. discovered that targeting of the KIF4A/androgen receptor axis could be used to reverse endocrine therapy resistance in castration-resistant prostate cancer [31]. In this study, we confirmed that the up-regulation of KIF4A was associated with cell proliferation and migration in DU145 and LNCaP cells, suggesting that KIF4A may influence cell division and cell growth via modulating AR-mediated androgen signaling pathways. This hypothesis needs to be verified in further studies. KIF4A in glioblastoma and hepatocellular carcinoma has been found to be correlated with adverse prognostic biomarkers using bioinformation analyses [25,41], indicating that KIF4A expression is correlated with cancer malignancy. However, there is no definite link between KIF4A expression and apoptosis; therefore, identifying a potentially driver of KIF4A in PCa progression.

In conclusion, the up-regulation of KIF4A was correlated with the aggressiveness of PCa, BCR-free survival and disease-free survival. These findings suggest that KIF4A may be a potential diagnostic indicator and therapeutic target for PCa. Further studies are recommended to clarify the molecular mechanism of KIF4A in PCa and the predictive effect of KIF4A on postoperative prognosis.

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