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Original Article

Elevated expression of REV1 is a predictor of unfavorable prognosis in patients with prostate cancer

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Abstract: Background: DNA directed polymerase (REV1), a member of a new superfamily of DNA polymerases, contains highly conserved blocks of amino acid sequences. Accumulating studies have revealed that anomalous expression of REV1 may be linked with mutation frequencies of various cancer-related genes. However, at present, the clinical significance of REV1 in prostate cancer (PCa) has not been clarified. Methods: In this study, immunohistochemistry analysis was performed to analyze expression of REV1 proteins in human PCa and non-cancerous prostate tissues and further validated using The Cancer Genome Atlas (TCGA) dataset at the mRNA level. Subsequently, association of REV1 expression with cancer progression and prognosis in PCa patients was statistically analyzed. Results: REV1 expression was elevated in PCa tissues compared to normal and hyperplasia prostate tissues (P = 0.003). Statistically, increased expression of REV1 proteins was significantly associated with advanced pathological grade (P = 0.039), clinical stage (P = 0.022), enhanced tumor invasion (P = 0.035), and high Gleason score (P = 0.017), in line with results based on TCGA data. Moreover, Kaplan-Meier analysis revealed that high levels of REV1 had significantly shorter overall survival (P = 0.006) and biochemical recurrence (BCR)-free survivals (P = 0.045). Multivariate analysis by Cox regression indicated that REV1 was an independent predictor for short overall survival (HR: 9.604, 95% CI: 1.199-76.896; P = 0.033). Conclusion: This study's findings suggest that aberrant expression of REV1 may be associated with carcinogenesis and cancer progression in PCa patients. Importantly, elevated expression of REV1 may be a predictor of unfavorable prognosis in PCa patients.

Keywords: REV1, Pca, cancer progression, prognosis

Introduction

Prostate cancer (PCa) is the most commonly diagnosed malignancy in men, worldwide, and a major cause of cancer-related deaths [1]. Incidence of PCa in developed countries is higher than in developing countries. It is continuously increasing in many countries around the world [2]. Current inability to distinguish indolent PCa from progressive PCa poses a great challenge in deciding appropriate management strategies for treatment of this disease. Although serum prostate-specific antigen (PSA) levels have been used for early diagnosis of PCa [3], they lack specificity which leads to overtreatment of indolent diseases [4]. An increasing number of clinicopathological characteristics such as Gleason score (GS), TNM stage, surgical margin, lymph node status, and prostate-specific antigen (PSA), alone or in combination, have been commonly used to predict prognosis of PCa patients [5-7]. PSA testing is generally considered to benefit treatment and early diagnosis of PCa, but its true value remain controversial [7]. Routine PSA testing might lead to over-diagnosis, contributing to 23-42% of all diagnosed cases [8]. Over-diagnosed patients, receiving treatments like radical prostatectomy or radiotherapy, may pay substantial costs and suffer serious side effects [9]. Nevertheless, prognosis of PCa patients with the same indicator results can be completely different due to patient heterogeneity and the complex molecular mechanisms underlying

Table 1. Clinicopathologic characteristics of all patients

Clinical features	Experiment type	
	Immunohistochemistry	TCGA data
Prostate cancer	50	498
Mean Age	67.22±11.52	61.05±6.81
< 60	4	202
≥ 60	46	296
Gender		
Male	50	498
Gleason score		
< 7	18	44
= 7	9	248
> 7	21	206
Person Neoplasm Status		
Tumor free	-	317
With tumor	-	94
Pathological grade		
< 2	10	-
= 2	20	-
> 2	18	-
Clinical stage		
I-II	23	-
III	10	-
IV	17	-
Tumor invasion		
T1-T2	27	352
T3	18	53
T4	5	2
Lymph nodemetastasis		
N0	33	345
N1	17	80
Distant metastasis		
M0	31	456
M1	19	3
Benign	10	-
Hyperplasia	20	

Note: Some item information is missing for some patients. "-" means there was lack of relative information of patients in our cohort.

prostate carcinogenesis. Therefore, it is crucial to discriminate original and efficient diagnostic and prognostic markers for PCa, preventing overtreatment and improving patient quality of life [10].

The ability of cells to tolerate DNA damage is crucial to occurrence and progression of diseases and cancers. When DNA damage occurs during replication, it will interrupt the replication fork, eventually causing fork collapse and genome rearrangements [11, 12]. To avoid that, cells have evolved a translesion DNA synthesis (TLS) system to replicate damaged DNA templates [13]. Recent studies have reported that expression levels of various translesion synthesis genes may be increased in several human cancers including breast cancer, lung cancer, and gastric cancer, implying their involvement in carcinogenesis. Moreover, activation of TLS may promote acquired drug resistance in tumor cells treated with DNA-damaging anticancer agents [14].

REV1 (DNA directed polymerase), together with POLη, POLκ, and POLι, belongs to the Y-family DNA polymerases and functions as an essential partner of Polζ in TLS. Similar to Polζ, REV1 is required for DNA damage-induced mutagenesis. In an *in vivo* system, the catalytic activity of REV1 has been used during the bypass of several lesions [15-18], playing an important role in maintaining the structure of TLS. In an *in vitro* system, the nucleotidyl transferase activity of REV1 was limited to the incorporation of just one or two dCMP moieties in a template-directed manner, regardless of template nucleotide composition [19, 20]. In recent studies, several single nucleotide polymorphisms (SNPs) of the REV1 gene have been reported to be related to various human cancers. For example, REV1-257Ser allele was identified as a risk allele for lung adenocarcinoma and squamous cell carcinoma [21]. In some *in vitro* studies, cultured human cell lines showed that using short hairpin RNA to inhibit REV1 reduced the rate of emergence of cisplatin resistance [22, 23]. However, the involvement of REV1 in human PCa remains unclear.

To address this problem, in the current study, expression patterns and subcellular localization of REV1 proteins were examined by immunohistochemistry based on human tissue microarray (TMA). Association of REV1 expression with various clinicopathological characteristics and patient prognosis were also statistically evaluated.

Materials and methods

Patients and tissue samples

For immunohistochemistry analysis, tissue microarrays (TMA, n = 80) including 50 PCa tissues, 20 hyperplasia tissues, and 10 adjacent

normal prostate tissues were obtained from Xi'an Alenabio Co, LTD (Cat No: PR807a, xi'an, China). No patients received chemotherapy or radiotherapy before the surgery. For survival analysis, expression levels of REV1 mRNA and clinical information of 498 PCa tissues were collected from TCGA database (https://tcga-data.nci.nih.gov/docs/publications/tcga/). Detailed information on the clinicopathologic characteristics of all patients used in this study are provided in **Table 1**.

Immunohistochemistry analysis

Tissue specimens were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. The paraffin-embedded tissue specimens were cut at 4 μm. They were then deparaffinized with xylene and rehydrated for further peroxidase (DAB) immunohistochemistry staining using DAKO EnVision System (Dako Diagnostics, Switzerland). Following brief proteolytic digestion, using an IHC enzyme antigen retrieval agent (Boster Biological Technology, Ltd., Wuhan, China) and peroxidase blocking blocking with 3% H_2O_2, the slides were incubated overnight at 4°C with the primary antibody against REV1 (Rabbit polyclonal antibody, DF2581, Affinity Biosciences, Inc., USA) at a dilution of 5 μg/mL. After washing with PBS, peroxidase labeled polymer and substrate-chromogen were employed to visualize the staining of REV1 proteins. Negative controls were carried out by omitting the primary antibody.

Evaluation of immunostaining results

Immunostaining results were scored, separately, by two independent experienced pathologists blinded to the clinicopathologic characteristics and clinical outcomes of PCa patients. The scores of the two pathologists were compared and any discrepant scores were reevaluated by both pathologists to achieve a consensus. The immunoreactive score (IRS) was calculated based on immunostaining intensity and the percentage of cells with positive immunostaining. Immunostaining intensity was scored according to the following criteria: no staining (0), weak staining (1), moderate staining (2), and strong staining (3). Percentage of positive-staining cells was calculated according to the number of positive-staining cells counted in five representative fields. Percentage scoring of immunoreactive cells was defined as follows: < 5% (0), 6-25% (1), 26-50% (2), 51-75% (3), and > 75% (4). The final IRS of each case was calculated as the sum of the two scores of immunostaining intensity and immunostaining percentage. Protein expression was further divided into high level (IRS ≥ 4) and low level (IRS < 4), based on the median IRS value.

Statistical analysis

SPSS 22.0 software (SPSS, Armonk, NY, USA) was used for statistical analysis. Pearson's Chi-square test and Fisher's exact test were used to evaluate associations of REV1 protein expression in TMA with various clinicopathological characteristics of PCa patients. Student's t-test was used to evaluate association of REV1 mRNA expression in TCGA with various clinicopathological characteristics of PCa patients. Survival curves were plotted using the Kaplan-Meier method and compared using log-rank test. Survival data were evaluated using univariate and multivariate Cox proportional hazards regression analyses. Relative risks of dying were shown as adjusted hazard ratios (HRs) and corresponding 95% confidence intervals (CIs). Differences were considered statistically significant when $P < 0.05$.

Results

Elevated expression of REV1 protein in human PCa tissues

Moderately positive REV1 immunostainings were observed in the cytoplasm in cancer cells of PCa tissues, but weakly in normal prostate and hyperplasia tissues. Of 49 PCa tissues, 16 (32.7%) showed low REV1 expression and 33 (67.3%) highly expressed REV1. In addition, there were 11 (55%) and 9 (45%) hyperplasia prostate tissues with low and high REV1 expression, respectively. Moreover, only 1 (10%) of normal prostate tissue exhibited high REV1 expression. Statistically, expression levels of REV1 proteins in PCa tissues were significantly higher than in non-cancerous prostate tissues (IRS: PCa = 4.08±1.30 vs. Normal = 2.50±0.71 $P < 0.001$; PCa = 4.08±1.30 vs. Hyperplasia = 3.15±1.14, P = 0.007) (**Figure 1B**).

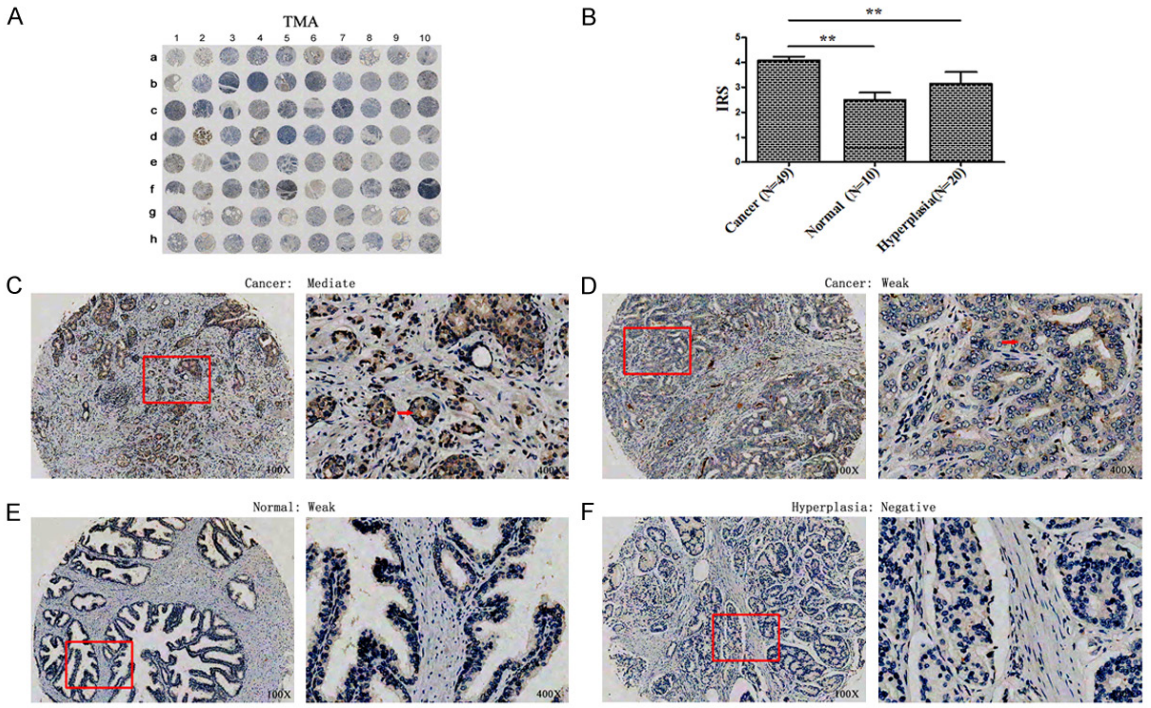

Figure 1. Immunostaining of REV1 protein in prostate cancer, normal prostate, and hyperplasia tissues. (A) Immunostaining of TMA from 80 samples. (B) IRSs of REV1 protein in Prostate cancer, normal prostate, and hyperplasia tissues. Expression levels of REV1 protein in Prostate cancer tissues were significantly higher than that in non-cancerous prostate tissues (IRS: Prostate cancer = 4.08±1.30 vs. Normal = 2.50±0.71, P < 0.001; Prostate cancer = 4.08±1.30 vs. Hyperplasia = 3.15±1.14, P = 0.007). **mean P < 0.01; (C, D) Representative immunohistochemical photographs of REV1 protein in Prostate cancer tissues. (E, F) Representative immunohistochemical photographs of REV1 protein in normal and hyperplasia prostate tissues. The red arrows in (C, D) show positive staining cells.

Elevated expression of REV1 protein and mRNA associates with aggressive progression of PCa patients

Pearson's Chi-square test showed that elevated expression of REV1 protein was significantly associated with high Gleason score (P = 0.017, **Table 2**), advanced pathological grade (P = 0.039, **Table 2**), clinical stage (P = 0.022, **Table 2**), and the presence of tumor invasion (P = 0.035, **Table 2**). However, there was no significant association of REV1 protein expression with patient age, lymph node metastasis, and distant metastasis (both P > 0.05) (**Table 2**).

To validate the results, a publicly available database (TCGA database) consisting of 498 PCa tissues with high-throughput sequencing data for protein coding genes (mRNA) expression data was used. Consistently, elevated expression of REV1 mRNA in PCa tissues was significantly associated with high Gleason score (P < 0.001), neoplasm status (P = 0.047), and positive distant metastasis (P = 0.003) of PCa patients. However, REV1 mRNA expression had no significant association with patient age, tumor invasion, and lymph node metastasis (all P > 0.05, **Table 2**).

REV1 is an independent prognostic marker of PCa patients

Association of REV1 mRNA expression with overall, BCR-free, non-metastatic, and non-metastatic BCR-free survivals of PCa patients were analyzed by Kaplan-Meier plots based on the TCGA database. As shown in **Figure 2**, PCa patients with high REV1 mRNA expression had shorter overall (P = 0.006), BCR-free (P = 0.045), non-metastatic (P = 0.009), and non-metastatic BCR-free (P = 0.041) survival than those with low REV1 mRNA expression. In addition, univariate analysis revealed that REV1 mRNA expression (HR 2.686, 95% CI 1.341-5.382; P = 0.005) was significantly associated with overall survival of PCa patients (**Table 3**). Furthermore, multivariate analysis using Cox proportional hazards model identified REV1

Table 2. Association of REV1 expression with clinicopathologic characteristics in prostate cancer patients

Clinical features	Case	TMA			TCGA		
		Low, n (%)	High, n (%)	*P*	Case	$\bar{x} \pm s$	*P*
Tissue							
Cancer	49	16 (32.7)	33 (67.3)	0.003**	498	835.21±181.60	-
Benign	10	9 (90.0)	1 (10.0)		-	-	
Hyperplasia	20	11 (55.0)	9 (45.0)				
Age							
< 60	4	3 (75.0)	1 (25.0)	0.829	202	831.53±185.56	0.839
≥ 60	45	14 (31.1)	31 (68.9)		296	834.96±185.62	
Gender							
Male	49	16 (32.7)	33 (67.3)	-	498	835.21±181.60	-
Female	-	-	-		-	-	
Gleason score							
< 7	18	7 (38.9)	11 (61.1)	0.017*	44	846.49±163.04	< 0.001**
= 7	9	6 (66.7)	3 (33.3)		248	800.30±190.09	
> 7	21	3 (14.3)	18 (85.7)		206	870.86±177.38	
Person Neoplasm Status							
Tumor free	-	-	-	-	317	814.23±185.76	0.047*
With tumor	-	-	-		94	856.62±165.06	
Pathological grade							
< 2	10	5 (50.0)	5 (50.0)	0.039*	-	-	-
= 2	20	9 (45.0)	11 (55.0)		-	-	
> 2	18	2 (11.1)	16 (88.9)		-	-	
Clinical stage							
I-II	23	12 (52.2)	11 (47.8)	0.022*	-	-	-
III	9	1 (11.1)	8 (88.9)		-	-	
IV	17	3 (17.6)	25 (82.4)		-	-	
Tumor invasion							
T1-T2	27	13 (48.1)	14 (51.9)	0.035*	352	818.19±189.36	0.210
T3	17	2 (11.8)	15 (88.2)		53	848.36±181.48	
T4	5	1 (20.0)	4 (80.0)		2	1008.65±201.04	
Lymph node metastasis							
N0	32	12 (37.5)	20 (62.5)	0.321	345	834.02±191.43	0.175
N1	17	4 (13.5)	13 (76.5)		80	865.35±159.80	
Distant metastasis							
M0	30	12 (40.0)	18 (60.0)	0.168	456	829.30±182.79	0.003**
M1	19	4 (21.1)	15 (78.9)		3	910.29±20.18	

Note: "-" refers to the missing information; *P < 0.05, **P < 0.01.

mRNA expression as an independent prognostic factor in PCa patients (HR 9.604, 95% CI 1.199-76.896; *P* = 0.033) (**Table 3**).

Discussion

Patients may have different clinical courses, with similar clinicopathological characteristics, when being treated with the same therapy. This suggests that current diagnostic markers are limited. Thus, it is important to find new biomarkers for treatment of PCa patients, establishing personalized treatment for each individual.

This present study is the first to investigate association between levels of REV1 and clinical features of PCa patients. In this study, there

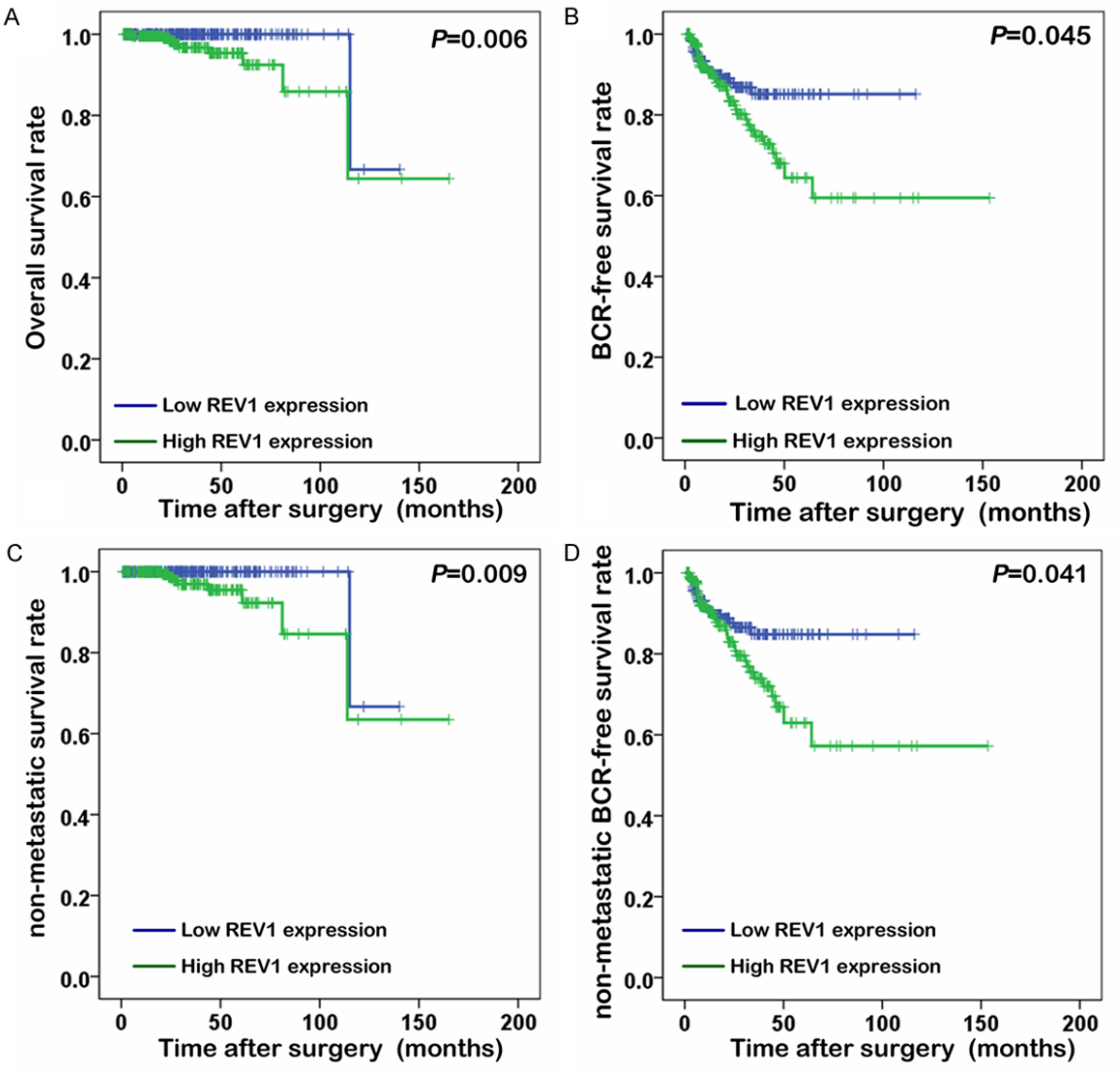

Figure 2. Kaplan-Meier curves of overall (A), BCR-free (B), non-metastatic (C), and non-metastatic BCR-free (D) survival based on REV1 mRNA expression in prostate cancer patients.

were three main findings. First, the IHC method was used to detect levels of REV1 in PCa patients. It was found that levels of REV1 were higher in PCa tissues than in adjacent non-cancerous or normal tissues. Second, this study described significant association of REV1 to clinical or pathological stage and personal neoplasm status, Gleason score, and distant metastasis. Third, this study showed that levels of REV1 were significantly associated with overall and BCR-free survivals of PCa patients. Kaplan-Meier analysis showed that overexpression of REV1 tended to have a significantly shorter overall and BCR-free survival, indicating that high levels of REV1 are biomarkers of poor prognosis for overall and BCR-free survival of PCa patients. Multivariate analyses show that upregulation of REV1 is a predictor of shorter overall survival, independent of Gleason scores. Results from this present study suggest that REV1 may play an important role in the aggressiveness of PCa, providing useful information helping clinicians establish personalized treatment for patients.

REV1, a member of a new superfamily of DNA Polymerases, involves highly conserved blocks of amino acid sequences [24]. It exerts template-dependent (but not template-directed) intrinsic DNA polymerase activity [20] and may be associated with bypassing various types of DNA lesions, albeit with remarkably limited abil-

Table 3. Prognostic value of REV1 mRNA expression for overall survival of prostate cancer patients using Cox proportional hazards model

Variable	Overall survival	
	HR (95% CI)	*P*
Univariate analysis		
Person Neoplasm Status (With tumor vs. Tumor free)	8.758 (1.689-45.416)	0.010*
Age (≥ 60 vs. < 60)	1.196 (0.336-4.259)	0.782
Gleason score (< 7 vs. = 7 vs. > 7)	5.996 (1.310-27.440)	0.021*
Tumor invasion (T1-T2 vs. T3 vs.T4)	6.782 (2.383-189.301)	< 0.001**
Lymph Node Stage (N0 vs. N1)	3.461 (0.765-15.655)	0.107
Distant metastasis (M0 vs. M1)	59.667 (6.550-543.565)	< 0.001**
REV1 expression (low vs. high)	2.686 (1.341-5.382)	0.005**
Multivariate analysis		
Age (≥ 60 vs. < 60)	1.566 (0.405-6.048)	0.515
Distant metastasis (M0 vs. M1)	39.608 (3.778-415.239)	0.002**
REV1 expression (low vs. high)	9.604 (1.199-76.896)	0.033*

Note: *P < 0.05, **P < 0.01.

ities. Accumulating studies have reported that REV1 protein may be related with mutation frequencies of various cancer-related genes [25]. It is also an important determining factor of DDP-induced genomic instability in human ovarian carcinoma cell [26].

The co-immunoprecipitation results of Narottam et al. indicated that REV1 physically associates with Polζ. This interaction may enhance the proficiency of Polζ for extension and mismatch extension from DNA lesion-bearing primer termini [27]. REV1 expression has also been observed to be significantly enhanced in glioma, compared with normal brain tissue, and has been positively correlated with the pathologic grade of glioma patients. Similarly, the data in this study showed elevated expression of REV1 in PCa tissues compared to non-cancerous prostate tissues. Statistically, it was demonstrated that both REV1 protein and mRNA overexpression were observably associated with aggressive progression of PCa patients. Moreover, elevated REV1 expression efficiently predicted unfavorable prognosis in PCa patients, including overall, BCR-free, non-metastatic, and non-metastatic BCR-free survival. This study also identified REV1 expression as an independent prognostic factor of overall survival of PCa patients.

This study demonstrates that REV1 has an obvious function in PCa progression. However, the mechanism by which REV1 acts in PCa remains unclear. As a member of translesion DNA synthesis (TLS) family of specialized DNA polymerases, REV1 is responsible for the majority of spontaneous and DNA damage-induced mutagenesis, *in vivo* [28-30]. It can co-localize with proliferating cell nuclear antigens (PCNA) in replication factories [31] and bind with monoubiquitinated PCNA in cells exposed to UV radiation [32]. REV1 is believed to function as a scaffold protein for polymerase switching at sites of lesions during TLS [33]. Beyond its primary role in TLS, REV1 can localize at regions near double-strand breaks (DSBs) in budding yeast [34]. DSBs initiate diverse responses, including homologous recombination (HR), which in eukaryotes mainly results in gene conversion. Therefore, it is possible that REV1 promotes malignant development in PCa by recruiting at DSBs to impact DNA repair.

In conclusion, the findings of this present study suggest that aberrant expression of REV1 may be associated with carcinogenesis and cancer progression in PCa patients. Importantly, elevated expression of REV1 may be a predictor of unfavorable prognosis for PCa patients. Since there are likely various regulators or effectors of REV1, the molecular mechanisms underlying REV1 involvement in PCa should be further explored in future studies.

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Disclosure of conflict of interest

None.

Abbreviations

REV1, DNA directed polymerase; PSA, Prostate specific antigen; TMA, Tissue microarray; BCR, Biochemical recurrence; TCGA, The Cancer Genome Atlas.

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