Elevated XPO6 expression as a potential prognostic biomarker for prostate cancer recurrence

Jun Hao[1,2,4], Yan Ting Chiang[1,2,4], Peter W. Gout[1], Yuzhuo Wang[1,2,3]

[1]Department of Experimental Therapeutics, BC Cancer Agency, Vancouver, BC, Canada, V5Z 1L3, [2]Vancouver Prostate Centre, Vancouver, BC, Canada, V6H 3Z6, [3]Department of Urologic Sciences, Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada, V5Z 1M9, [4]Interdisciplinary Oncology Program, Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada, V5Z 1L3

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1. ABSTRACT

Recurrence of localized prostate cancer following treatment can lead to lethal metastatic castration-resistant prostate cancer. Although numerous studies aimed at developing biomarkers for predicting recurrence of localized prostate cancer are promising, they have not yet led to useful applications. Dysregulation of exportins (XPOs, nucleocytoplasmic transporters) associated with subcellular mislocalization of proteins has been reported for various human cancers. However, most of the XPOs have not been studied in prostate cancer. In this study, we are the first to examine whether changes in expression of XPOs could be used as potential biomarkers for recurrence of localized prostate cancer. Using the oncomine database, gene expressions of 7 known XPOs by 1128 patient samples, obtained from 16 independent prostate cancer patient cohorts, were analyzed. Relatively highly elevated expression of *XPO6* (compared to prostate cancer tissue) was found to be significantly associated with poor patient prognosis, in particular, with rapid recurrence in a clinical "low risk" group. As such, expression of XPO6 may be a potential prognostic biomarker for predicting prostate cancer recurrence.

2. INTRODUCTION

Prostate cancer is the most commonly diagnosed non-cutaneous cancer and a leading cause of cancer death for North American men (1). When the malignancy is localized to the prostate, surgery and radiation therapy can be curative. However, many treated patients will experience local recurrence which can lead to metastatic cancer for which there is currently no cure (2). Currently, one of the most commonly used strategies for recurrence risk prediction is the D'Amico risk stratification which is based on the initial PSA level, biopsy Gleason score and clinical T stage (3-5). However, this method has limited predictive power leading to either overtreatment or undertreatment (6,7). Recently, studies aimed at developing biomarkers for predicting prostate cancer progression have made promising progress in the laboratory (8-21). However, very few biomarkers have proven useful in the clinic. Clearly, discovery and development of new reliable biomarkers to predict cancer recurrence is urgently required for improving disease management and patient survival.

In eukaryotic cells, proteins made in the cytoplasm need to be transported to various subcellular locations, such as the nucleus, to fulfill their particular functions. Proper localization of proteins is of major importance for normal functioning of cells. The transportation of the proteins is mediated by karyopherins. Proteins also need to be exported from the nucleus and, in such a case, their localization is mediated by karyopherins known as exportins (XPOs) (22). Exportins are proteins which can identify and

bind to a cargo via recognition of a specific nuclear export signal (NES); they share a common N-terminal domain. To date, 7 XPOs have been identified in humans, each of them being responsible for exporting specific molecules from the nucleus to the cytoplasm (22). Dysregulation of XPOs associated with subcellular mislocalization of proteins has been reported for various types of human cancer(23). Upregulation of certain XPOs in particular has been associated with cancer progression (24-32).

The present study was aimed at (i) investigating changes in the gene expression of XPOs in prostate cancer, and (ii) determining whether the changes could be used as potential biomarkers for the recurrence of localized prostate cancer. Using the oncomine database, we examined the expressions of *XPOs* in data from a total of 1128 patient samples obtained from 16 independent clinical cohorts (8, 10, 33-46). We found that *XPO6* was elevated in primary prostate cancer tissues as well as metastatic tissues and that its elevated expression correlated with increased prostate cancer aggressiveness, suggesting that the XPO6 protein can provide a novel, prognostic biomarker for prostate cancer recurrence.

3. MATERIALS AND METHODS

3.1. Oncomine database analysis

Gene expression data of 7 XPOs were obtained from 16 different prostate cancer cohorts (8, 10, 33-46) using the Oncomine database (47). Expression values of XPOs are presented in $\log_2$ median-centered intensity values for each study.

3.2. Prognostic value analysis

Gene expression data from 131 primary prostate cancer tissue specimens and the biochemical recurrence-free survival times of the patients (acquired from NCBI GEO under accession GSE21032)(42) were analyzed using Kaplan-Meier analysis and Cox proportional-hazards regression analysis. Clinical and pathologic data (patient age, tumor site, PSA level, T stage, Gleason score, metastasis, biochemical recurrence, time until biochemical recurrence) were also collected (Table 5) for validating the prognostic values of the XPOs.

3.3. Statistical analysis

P<0.0.5 was used as the significant threshold level unless otherwise mentioned. Significance comparisons between 2 different groups were calculated using the Student's t test. GraphPad Prism software (Version 4.0.3, GraphPad Software Inc., La Jolla, CA) was used for Kaplan-Meier analysis and the log-rank test was used to determine the difference between curves. Univariate and multivariate Cox proportional-hazards regression models were analyzed by SigmaPlot software (Bersion 11.0., Systat Software Inc., San Jose, CA) and the significance values and odds ratios were calculated by the likelihood ratio test. Both analyses were used

to evaluate the association of various factors with biochemical recurrence. The level of significance in the statistical analyses is indicated as *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P<0.0001.

4. RESULTS

4.1. Elevated expression of *XPO3* and *XPO6* in prostate cancer

To reveal potential differential expressions of the XPOs in prostate cancer, we compared the expression of the XPOs in prostate cancer tissues and normal prostate tissues from 16 independent prostate cancer patient cohorts (8, 10, 33-46) using the same cut-offs (P value<0.0.1, gene rank top/bottom 10%, Table 1). In none of these cohorts a significant difference was found in *XPO1* or *XPO5* expression between normal and cancer tissues. Elevated expression of *XPO4* and *XPO2* was found only in 1 and 3 cohorts, respectively. There was no significant change in the expression of *XPO7* as there were conflicting results between the various cohorts. The expressions of *XPO3* and *XPO6* were markedly and consistently upregulated in 8 and 7 cohorts, respectively (Figure 1A, B). *XPO3* had a higher expression in prostate cancer tissues compared to normal tissues in 8 cohorts with fold changes varying between 1.34 to 1.96 and a significant P value (1.13E-9 to 6.00E-3). *XPO6* expression was elevated in 7 different cohorts with fold changes between 1.14 to 2.28 and significant P value (4.01E-5 to 2.00E-3).

4.2. Elevated *XPO6* expression is correlated with poor patient prognosis

We determined whether there was a correlation between the expression of *XPO3* and *XPO6* and poor patient prognosis (Table 2). *XPO6* expression was significantly higher in patients with elevated PSA levels (>20 ng/ml) prior to radical prostatectomy (P=0.02). Elevated *XPO6* expression was also found in patients with higher combined Gleason score (≥8) in both biopsy and radical prostatectomy specimens (P=1.34E-3, P=3.09E-3, respectively). Furthermore, a positive association was found for the elevated expression of *XPO6* and lymph node metastasis occurrence (P=0.01), biochemical recurrence (P=1.57E-3), and distant metastasis occurrence (P=3.27E-4), whereas there was no significant correlation between *XPO3* expression and poor prognosis or poor patient outcomes. Some cohorts showed that the expression of *XPO6* was higher in CRPC and metastatic prostate cancer compared to primary tumors (Figure 1C, D). Taken together, the results suggest that elevated expression of *XPO6* is significantly correlated with more aggressive prostate cancers and poor clinical outcomes.

4.3. Elevated *XPO6* expression is associated with biochemical recurrence of prostate cancer

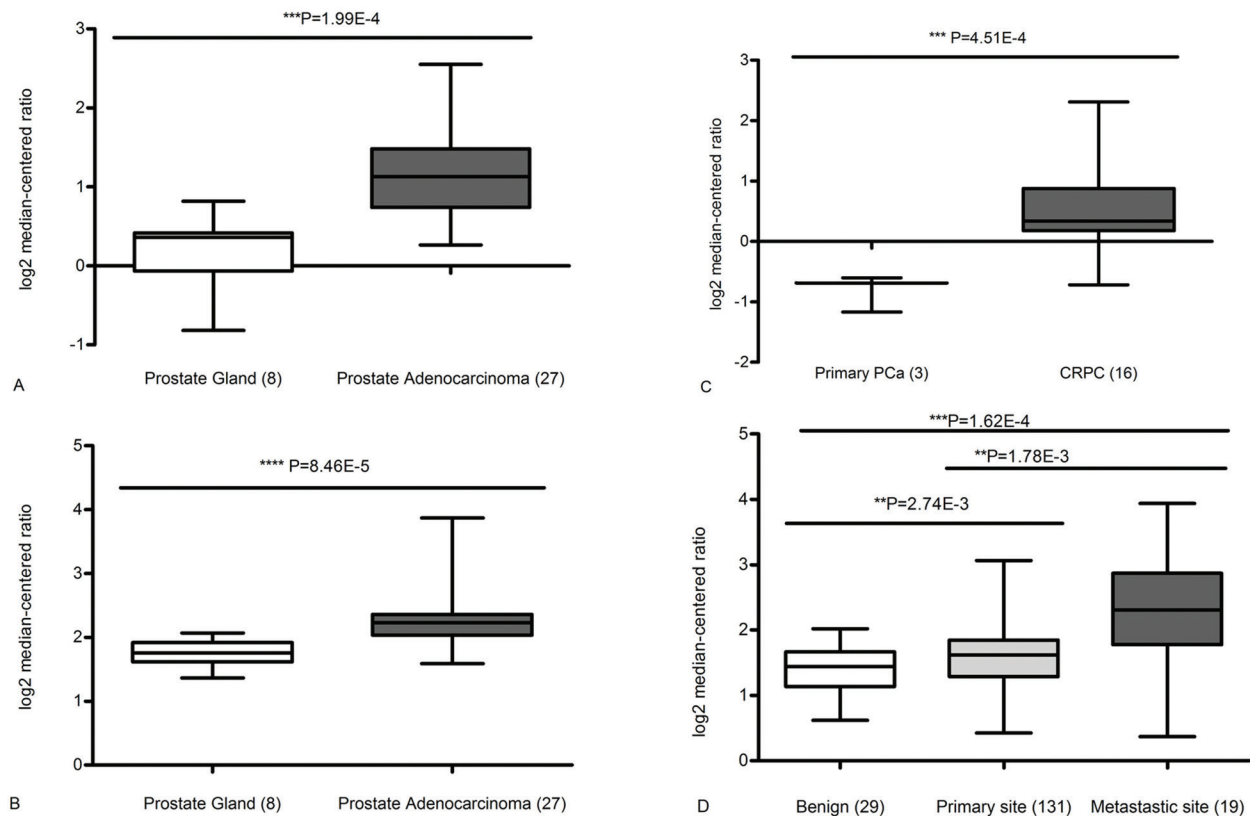We further focused on the correlation between the elevated expression of *XPO3* and *XPO6* and

Figure 1. *XPO3* and *XPO6* are differentially expressed in prostate cancer and normal prostate gland. (A) Elevated *XPO6* expression in prostate cancer samples compared to normal prostate gland using data from prostate cancer cohort published by Vanaja *et al*. (45). (B) Elevated *XPO3* expression in prostate cancer samples compared to normal prostate gland using data from prostate cancer cohort published by Vanaja *et al*. (45). (C) Elevated *XPO6* expression in primary prostate cancer samples compared to CRPC samples using data from prostate cancer cohort published by Tomlins *et al*. (8). (D) Elevated *XPO6* expression in metastatic prostate cancer samples compared to primary prostate cancer samples and benign prostate gland samples using data from prostate cancer cohort published by Taylor *et al*. (42). Sample numbers are shown in brackets. CRPC: castration-resistant prostate cancer.

Table 1. Differential gene expression of the XPOs in prostate cancer compared to normal prostate tissue

Genes	Studies (up/total)	Studies (down/total)	Sample number	P value	Fold change	Gene rank (%)	Reference
XPO1	0/15	0/15	-	-	-	-	-
XPO2	3/16	0/16	82	5.00E-5 – 7.09E-4	1.13 – 1.53	3.94 – 9.62	38, 45, 51
XPO3	8/15	0/15	495	1.13E-9 – 6.00E-3	1.34 – 1.96	0.80 – 7.59	37, 38, 42, 44, 45, 47, 49, 51
XPO4	1/10	0/10	21	2.00E-3	1.63	4.30	45
XPO5	0/8	0/8	-	-	-	-	-
XPO6	7/15	0/15	461	4.01E-5 – 2.00E-3	1.14 – 2.28	3.48 – 9.84	37, 44, 45, 47, 48, 50, 51
XPO7	1/15	3/15	89/74	1.06E-4/(1.21E-4 – 1.00E-3)	3.10/(−1.23 − −1.93)	1.21/(2.37 – 3.98)	45, 46, 48, 52
Study details included in the supplementary materials (Supplementary Table 5-10)							

biochemical recurrence. Recurrence-free survival curves were calculated using the Kaplan-Meier analysis. The samples were grouped according to the expression levels, where high expression means samples with top 30% *XPO3* expression among all primary tumors. The expression of *XPO3* did not significantly correlate with biochemical recurrence (P=0.07, Figure 2A), as indicated by Kaplan-Meier analysis. The latter also
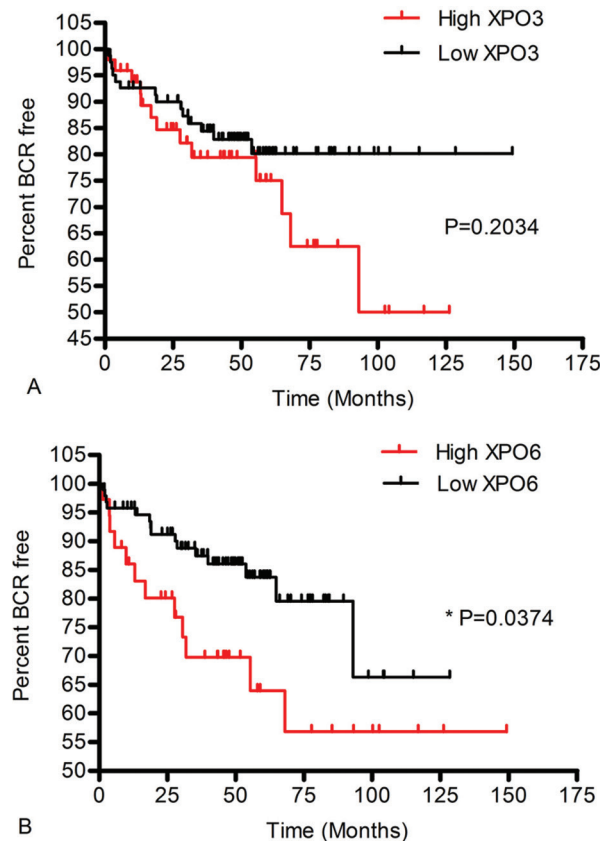
Figure 2. Kaplan-Meier time to recurrence curves for 131 prostate cancer samples from primary sites are shown. (A) Samples were grouped according to *XPO3* mRNA expression level (P=0.204, Hazard ratio=1.52, 95% CI of ratio=0.76 to 3.65). High *XPO3* represents samples with top 30% *XPO3* expression among all primary tumors. (B) Samples were grouped according to *XPO6* mRNA expression level (P=0.04, Hazard ratio=2.18, 95% CI of ratio=1.05 to 5.85). High *XPO6* represents cases with *XPO6* expression Z-Score>1.5. compared to normal. Significance levels were calculated using the log-rank test.

Table 2. Elevated *XPO6* expression is correlated with poor prognosis in 131 primary prostate cancer samples

Poor prognosis factor	P value	
	XPO3	*XPO6*
PSA <20 vs. PSA ≥20 ng/ml	0.39	0.02
Biopsy Gleason score <8 vs. Gleason score ≥8	0.47	1.34E-03
Radical prostatectomy Gleason score <8 vs. Gleason score ≥8	0.38	3.09E-03
N0 vs. N1 (Lymph node metastasis)	0.39	0.01
No recurrence after treatment vs. recurrence after treatment	0.45	1.57E-03
M0 vs. M1 (distant metastasis)	0.42	3.27E-04

indicates that patients bearing tumors with elevated expression of *XPO6* had a significant shorter time until recurrence (P=0.04, Hazard Ratio=2.19, 95% CI between 1.07 and 5.635, Figure 2B). The mean recurrence-free survival time of patients with elevated *XPO6* expression was 6.5. months shorter compared to other patients.

Using the Cox proportional-hazards regression method, we confirmed that the elevated expression of *XPO3* did not correlate with recurrence-free survival time (OR=0.44, P=0.50, Table 3), whereas there was a significant correlation between the elevated expression of *XPO6* and biochemical recurrence (Odds Ratio=7.32, P=6.81E-3, Table 3). The combination of using *XPO6* expression as a predictive factor and the D'Amico stratification gave a better prognostic prediction value (OR=14.04, P=8.93E-4) than when these approaches were used on their own (OR=7.32, P=6.81E-3; OR=10.11, P=1.47E-3, respectively). Both *XPO6* expression and D'Amico stratification contributed significantly to this combination (XPO6: HR=2.9.7, 95% CI between 1.04 and 8.51, P=0.04; D'Amico: HR=2.96, 95% CI between 1.34 and 6.53, P=7.35E-3, Table 4).

4.4. *XPO6* as a prognostic biomarker in a "low risk" patient group

To explore whether the expression of *XPO6* can benefit current prostate cancer risk stratification, we focused on patients who were grouped at the time of diagnosis as "low risk" based on D'Amico risk stratification. 60 primary samples were grouped as "low risk" and 8 of them were found to have a biochemical recurrence. Using the Cox proportional-hazards regression method, we found that elevated expression of *XPO6* significantly correlated with biochemical recurrence (P=0.02, Odds Ratio=5.61). To confirm this, we separated patients into two groups according to "High *XPO6*" (*XPO6* expression 1.5. fold higher than the median) and "Low *XPO6*" (the remaining samples), and subjected them to Kaplan-Meier analysis. As shown in Figure 3, the two groups of patients had significantly different times until recurrence (P=2.70E-7, Hazard ratio=15.79, 95% CI of the ratio 423.4.-7.26E5). These results indicate that *XPO6* expression may be used as a novel biomarker for identification of potential "high risk" patients in a clinical low risk group.

5. DISCUSSION

Cancer recurrence following therapy of localized prostate cancer is a first indicator showing that a cancer may gradually develop into a lethal, metastatic CRPC. The risk stratification system currently used to predict cancer recurrence following therapy of localized prostate cancer lacks predictive ability and there is a
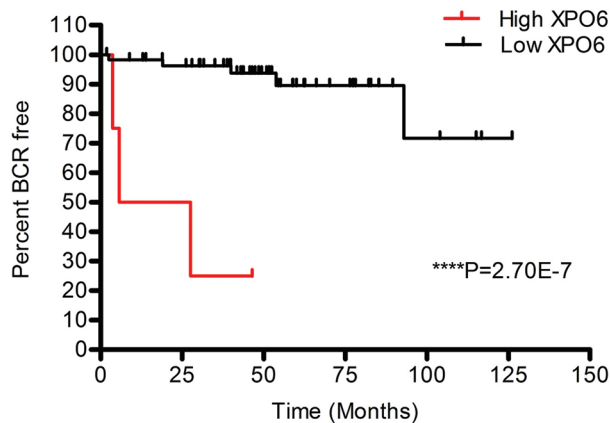
Figure 3. Kaplan-Meier time to recurrence curves for 60 low risk group patients are shown. Patients were grouped based on their *XPO6* expression level. Cases with *XPO6* expression 1.5. fold higher than the median were considered as high *XPO6* and shown in red. The difference between two curves was analyzed by log-rank test. (P=2.70E-7, Hazard ratio=15.79, 95% CI of the ratio 423.4-7.26E5).

Table 3. Potential prognostic value as indicated by Cox proportional-hazards regression analysis

	XPO3	*XPO6*	D'Amico	*XPO6*+D'Amico
OR[1]	0.44	7.32	10.11	14.04
Df[2]	1	1	1	2
P	0.50	6.81E-3	1.47E-3	8.93E-4

[1]Odds ratio, [2]degrees of freedom. Significance levels were calculated using the likelihood ratio test

Table 4. Contributions of *XPO6* expression and D'Amico in the combination group

	B[1]	SE[2]	Wald[3]	P	HR[4]	95% CI-L[5]	95% CI-U[6]
XPO6	1.09	0.54	4.12	0.0424	2.97	1.04	8.51
D'Amico	1.08	0.40	7.19	7.35E-3	2.96	1.34	6.53

[1]Coefficient, [2]Standard error, [3]Wald Chi-Square, [4]Hazard ratio, [5]95%CI-L: 95% confidence interval lower limit, [6]95%CI-U: 95% confidence interval upper limit

cancer aggressiveness and poor patient prognosis, as indicated by elevated blood PSA levels, increased Gleason score, biochemical recurrence, and lymph node/distant metastases. This suggests that use of relatively highly elevated *XPO6* expression (compared to prostate cancer tissue) as a potential biomarker for predicting poor prognosis, following therapy of localized prostate cancer, has clinical relevance.

Recently, various studies have suggested that the expression of both protein-coding and non-protein-coding genes can be used as potential biomarkers for the prediction of recurrence following therapy of localized prostate cancers (50-59). The majority of the studies, however, failed to discuss how such potential biomarkers could benefit the currently used clinical risk stratification system. In the present study, it is suggested that use of relatively highly elevated expression of *XPO6* (compared to prostate cancer tissue) as a prognostic biomarker could be particularly useful for identification of cancer recurrence in "low risk" patients (see Figure 3). If a "low risk" patient is shown to have relatively highly elevated expression of *XPO6*, he could be recommended for more aggressive treatment than normally used for localized prostate cancer, such as androgen deprivation therapy. Thus, relatively highly elevated expression of *XPO6* may be particularly useful as a biomarker in combination with the D'Amico risk stratification system to determine which treatment option should be selected for an individual patient. However, the Taylor patient cohort (42) used in the present study is the only publically available prostate cancer patient cohort with detailed patient clinical and pathologic information. Other cohorts are either limited in the number of patients or lack complete clinical and pathologic information. This obstacle weakens the findings since the clinical relevance was both found and validated using the same patient cohorts. To achieve better clinical relevance and strengthen the findings, more patient samples need to be analyzed when more patient cohorts are available.

Although the expression of *XPO3* was also found to be elevated in prostate cancer, it had poor clinical relevance as there was no correlation with prostate cancer progression. In contrast to a report suggesting that *XPO1* expression was elevated in prostate cancer cell lines (60), the present study did not show a statistically significant difference in *XPO1* expression between prostate cancer and normal prostate tissue. This discrepancy may be due to an inability of *in vitro* systems to accurately reflect tumor physiology (61).

In conclusion, relatively highly elevated expression of *XPO6* (compared to prostate cancer tissue) may provide a prognostic biomarker for identifying patients with high risk of developing recurrence following therapy of localized prostate cancer, in particular for

critical need for reliable prognostic biomarkers (48). In developing a candidate biomarker, it is of paramount importance that it has clinical relevance. Thus the majority of biomarkers that successfully pass preclinical tests fail when they are used in clinical trials (49). Using gene expression data and clinical data from patient cohorts we have, in the present study, shown that the expression of *XPO6* was significantly upregulated in prostate cancers. Furthermore, substantially elevated expression of *XPO6* correlated with increased prostate

Table 5. Clinical and pathological characteristics of patients used for the prognostic study

Parameters	Total	%
N	150	100.00
Tumor samples from primary site	131	87.33
Tumor samples from metastatic site	19	12.67
Age at diagnosis/median (range)	58.00 (37.30-83.00)	
PSA at diagnosis/median (range)	6.30 (1.09-506.00)	
Biopsy Gleason score		
5	1	0.67
6	79	52.67
7	50	33.33
8	10	6.67
9	9	6.00
Not available	1	0.67
Clinical T stage		
T1C	80	53.33
T2A	25	16.67
T2B	20	13.33
T2C	13	8.67
T3A	6	4.00
T4	1	0.67
Not available	5	3.33
PSA level prior to radical prostatectomy/median (range)	6.60 (1.15-506.00)	
Radical prostatectomy Gleason score		
6	41	27.33
7	76	50.67
8	11	7.33
9	11	7.33
Not available	11	7.33
Pathology T stage		
T2A	9	6.00
T2B	48	32.00
T2C	29	19.33
T3A	30	20.00
T3B	13	8.67
T3C	4	2.67
T4	8	5.33
Not available	9	6.00
Biochemical recurrence		
Yes	36	24.00
Time until biochemical recurrence (months)/median (range)	45.45 (1.38-149.19)	
No	104	69.33
Not available	10	6.67
Metastasis resulting from the primary tumor		
Yes	28	18.67
No	122	81.33

Table 6. *XPO2* differential expression studies in prostate cancer

XPO2	Study number	Study names	Sample number	P value	Fold change	Gene rank %	Reference
PCa[1] > normal	3	LaTulippe	26	5.00E-3	1.26	3.94	(34)
		Vanaja	35	7.09E-4	1.53	7.65	(45)
		Arredouani	21	8.00E-3	1.13	9.62	(40)
PCa[1] < normal	0	-	-	-	-	-	-
[1]Prostate cancer							

Table 7. *XPO3* differential expression studies in prostate cancer

XPO3	Study number	Study names	Sample number	P value	Fold change	Gene rank %	Reference
PCa[1] > normal	8	Grasso	87	1.13E-9	1.42	0.80	(43)
		Lapointe	103	7.75E-9	1.37	2.62	(39)
		Luo	30	5.00 E-3	1.38	1.80	(38)
		LaTulippe	26	6.00 E-3	1.36	4.07	(34)
		Welsh	34	3.24E-4	1.40	7.59	(33)
		Arredouani	21	2.00 E-3	1.52	4.18	(40)
		Vanaja	40	1.99E-4	1.96	4.72	(45)
		Taylor	160	6.51E-5	1.34	3.94	(42)
PCa[1] < normal	0	-	-	-	-	-	-
[1]Prostate cancer							

Table 8. *XPO4* differential expression studies in prostate cancer

XPO4	Study number	Study names	Sample number	P value	Fold change	Gene rank %	Reference
PCa[1] > normal	1	Arredouani	21	2.00 E-3	1.63	4.30	(40)
PCa[1] < normal	0	-	-	-	-	-	-
[1]Prostate cancer							

Table 9. *XPO6* differential expression studies in prostate cancer

XPO6	Study number	Study names	Sample number	P value	Fold change	Gene rank %	Reference
PCa[1] > normal	7	Tomlins	52	1.29E-04	2.28	4.79	(8)
		Welsh	34	9.21E-05	1.41	5.57	(33)
		Vanaja	35	8.46E-05	1.44	3.48	(45)
		Liu	57	2.00 E-3	1.14	5.05	(44)
		Lapointe	102	4.01E-05	1.27	8.97	(39)
		Arredouani	21	5.00 E-3	1.45	7.17	(40)
		Taylor	160	3.00 E-3	1.16	9.84	(42)
PCa[1] < normal	0	-	-	-	-	-	-
[1]Prostate cancer							

Table 10. *XPO7* differential expression studies in prostate cancer

XPO7	Study number	Study names	Sample number	P value	Fold change	Gene rank %	Reference
PCa[1] > normal	1	Wallace	89	1.06E-4	3.10	1.21	(46)
PCa[1] < normal	3	Tomlins	40	1.21E-4	-1.93	2.37	(8)
		Varambally	13	1.00E-3	-1.50	3.44	(41)
		Arredouani	21	1.00E-3	-1.23	3.98	(40)
[1]Prostate cancer							

patients with "low risk" of recurrence based on the D'Amico risk stratification system.

6. ACKNOWLEDGEMENTS

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Abbreviations: PSA: prostate specific antigen; NPCs: nuclear pore complexes; XPOs: exportins; NES: nuclear export signal; AR: androgen receptor; TP53: tumor protein p53; BRCA1: breast cancer1; XPO1: exportin 1; XPO2: CSE1L, CSE1 chromosome seqregation 1-like; XPO3: XPOT, exportin tRNA; XPO4: exportin 4; XPO5: exportin 5; XPO6: exportin 6; XPO7: exportin 7; CRPC: castration-resistant prostate cancer; OR: Odds ratio; HR: Hazard ratio; CI: Confidence interval

Key Words: Prostate Cancer, Prognostic Biomarker, Cancer Recurrence, Exportin 6

Send correspondence to: Yuzhuo Wang, Department of Experimental Therapeutics, BC Cancer Research Centre, 675 West 10th Avenue, Vancouver, BC, Canada, V5Z 1L3, Tel: 604-675-8013, Fax: 604-675-8019, E-mail: ywang@bccrc.ca