Histol Histopathol (2014) 29: 535-542
DOI: 10.14670/HH-29.10.535

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Prognostic impact of NDRG2 and NDRG3 in prostate cancer patients undergoing radical prostatectomy

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Summary. Aim: To investigate the clinicopathologic significance of NDRG2 and NDRG3, and their involvement in recurrence-free survival (RFS) and overall survival (OS) of prostate cancer (PCa). Methods: NDRG2 and NDRG3 expression in 206 pairs of primary PCa and corresponding noncancerous prostate tissue samples from the same specimens were detected by immunohistochemistry. The association of NDRG2 and NDRG3 expression with the clinicopathologic features and with the prognosis of PCa was subsequently assessed. Results: In PCa tissues, NDRG2 expression was significantly downregulated, while NDRG3 expression was significantly upregulated (both $P<0.001$), compared with those in corresponding noncancerous prostate tissues. In addition, the downregulation of NDRG2 in PCa tissues was significantly correlated with advanced pathological stage ($P=0.001$), positive metastatic status ($P=0.001$) and high Gleason score ($P=0.003$), while the upregulation of NDRG3 in PCa tissues was significantly correlated with advanced pathological stage ($P=0.006$), positive metastatic status ($P=0.001$) and lymph node status ($P=0.002$). Furthermore, multivariate survival analysis showed low NDRG2 and high NDRG3 immunoreactivities were both significantly associated with short RFS and short OS in PCa independently of routine clinicopathological predictors. Conclusion: Our data offer convincing evidence for the first time that the aberrant expression of NDRG2 and NDRG3 may contribute to the malignant progression of PCa. More importantly, both the downregulation of NDRG2 and the upregulation of NDRG3 may be efficient prognostic indicators for PCa.

Key words: Prostate cancer; N-myc downstream regulated gene 2, N-myc downstream regulated gene 3, Clinical pathology, Prognosis

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Introduction

Prostate cancer (PCa) is the most common noncutaneous malignancy of the urinary system and is one of the leading causes of cancer-related deaths among males in the world (Parnes et al., 2013). It has been reported that nearly 300,000 new cases of PCa were diagnosed in the USA in 2011, accounting for 29% of all newly diagnosed cancers in men (MacVicar and Hussain, 2013). Similarly, the number of PCa patients has dramatically increased in China (Ilic et al., 2013). It is a multistep process involving carcinogenesis and the mechanisms influencing the progression and prognosis of PCa. Surgery and radiation therapy are effective for localized disease (Martin et al., 2013). However, no effective therapeutic strategy is yet available for recurrent or metastatic disease from failed surgery, radiation, chemotherapy, or hormonal therapy. The advent of prostate-specific antigen (PSA) screening has led to earlier detection of clinically localized PCa (Wilt and Ahmed, 2013). Unfortunately, accumulating studies have found that PCa patients with the equivalent PSA level could have various clinical outcomes because of the molecularly heterogeneous subtypes (Sheikh et al.,

2013). With these limitations, it is of great significance to identify more sensitive and more specific biomarkers which can provide valuable information for the diagnosis and prognosis in PCa.

The N-myc downstream regulated gene (NDRG) protein family consists of 4 members, NDRG1, NDRG2, NDRG3, and NDRG4, which are well conserved through evolution (Melotte et al., 2010). The AceView database from NCBI shows that NDRG family members have an NDR– an α/ß hydrolase (ABH) –fold region (Ellen et al., 2008). In addition to these two characters, NDRG members also contain some functional sites, such as phosphorylation sites, acetylation sites, ubiquitination sites and so on (Wang et al., 2003). The sequence differences between NDRG members are predominantly located in the N- and C-terminal regions, except for the C-terminal 5-amino acid residues, which are completely preserved in all 4 NDRG proteins. According to the sequences, the NDRG family may be divided into two subfamilies: one composing NDRG1 and NDRG3, with 67% homology, and the other, including NDRG2 and NDRG4 with 58% homology (Kovacevic and Richardson, 2006). Functionally, NDRG proteins are suggested to play important roles in various biological processes and pathogenesis. Especially in cancer, the aberrant expression, tumor suppressive, and oncogenic functions have been reported for the NDRG family members. In PCa, NDRG1 has been identified as a metastasis suppressor that is down-regulated in cancer and metastatic cells when compared to normal cells (Ghalayini et al., 2013). Yu et al. (2011) observed the decreased expression of NDRG2 gene in three PCa cell lines. Gao et al. (Gao et al., 2011) further demonstrated that NDRG2 overexpression may inhibit tumor growth and invasion, and may decrease bone destruction caused by PCa bone metastasis. Wang et al. (Wang et al., 2009) observed NDRG3 expression in both epithelial prostate cancer cells and prostatic stromal cells at both mRNA and protein levels. Overexpression of NDRG3 increased their growth rates and migration capabilities *in vitro* and promoted the growth of xenograft tumors in nude mice. Overexpression of NDRG3 in cells could upregulate the expression of angiogenic chemokines, such as CXCL1, CXCL3 and CXCL5, which mainly increase the angiogenesis and ultimately promote the growth of tumors. Taking into account the importance of NDRG members in tumorigenesis and tumor progression of PCa, much more study is required.

The aim of this study was to investigate the clinicopathologic significance of NDRG2 and NDRG3, and their involvement in recurrence-free survival (RFS) and overall survival (OS) of PCa.

Materials and methods

Patients and tissue samples

The study was approved by the Research Ethics Committee of Ministry of Public Health of China (IRB approval number: XYLL20130089). Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Two hundred and six pairs of primary PCa and corresponding noncancerous prostate tissue samples from the same specimens were collected from the School of Public Health, Central South University, from 1998 to 2008. The selection criteria were as follows: (i) a diagnosis of primary PCa; (ii) receiving radical prostatectomy; (iii) no patients had received radiotherapy, or chemotherapy before surgery. (iv) information on follow-up could be obtained; (v) complete records of the cases pre- and post-operation, and samples of the primary tissue, had been preserved. The histopathologic features of tumor specimens were classified according to the Gleason score and 2002 tumor-nodes-metastases (TNM) classification system. The clinicopathological information of the patients is shown in Table 1. For Western blot analysis, 20 pairs of fresh primary PCa and corresponding noncancerous prostate tissue samples were immediately immersed in RNAlater (Ambion, Inc., USA) after surgical resection, stored at 4°C overnight to allow thorough penetration of the tissue and then frozen at -80°C until use.

All 206 PCa patients were given a follow-up exam ranging from three to ten years. For the analysis of survival and follow-up, the date of prostatectomy was used to represent the beginning of the follow-up period. The primary analysis endpoint was recurrence-free survival (RFS). Other analysis endpoints were overall survival (OS). All the patients who died from diseases other than PCa or from unexpected events were excluded from the case collection.

Western blot analysis

The fresh tissues from patients with PCa were homogenated in a RIPA lysis buffer and centrifuged at 20,000 x g for 60 min at 4°C to pellet any precipitate. The protein concentration of the lysate was determined by the BCA assay. Western blot analyses were carried out according to the standard protocol using nitrocellulose membranes (Bio-Rad). For immunoblotting, membranes were incubated with the primary antibody (1:1,000) for 2 h, followed by 1-h incubation with a 1:1,000 dilution of horseradish peroxidase (HRP)-linked secondary antibody. Finally, the immunoreactive proteins were detected by enhanced chemiluminescence assay with HRP (Pierce). The primary antibodies were as follows: mouse monoclonal anti-NDRG2 antibody (Tago, Burlingame, CA), rabbit monoclonal anti-NDRG3 antibody (Cell Signaling Technology, Danvers, MA), mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology, USA). To confirm equal loading, GAPDH antibody was served as a control.

Immunohistochemistry analysis

The sub-cellular localization and expression patterns of NDRG2 and NDRG3 proteins in PCa and

corresponding noncancerous prostate tissues were detected by immunohistochemistry analysis using paraffin-embedded specimens from 206 patients with PCa. Briefly, the paraffin-embedded tissues were cut at 4 μm and then deparaffinized with xylene and rehydrated for further H&E or peroxidase (DAB) immunohistochemistry staining employing DAKO EnVision System (Dako Diagnostics, Zug, Switzerland). The slides were incubated with mouse monoclonal anti-NDRG2 antibody (Tago, Burlingame, CA) and rabbit monoclonal anti-NDRG3 antibody (Cell Signaling Technology, Danvers, MA) overnight at 4°C. After washing, peroxidase labeled polymer and substrate-chromogen were then employed in order to visualize the staining of the interested proteins.

Assessment of immunohistochemical staining was evaluated by two independent pathologists who were blinded to the clinicopathological parameters and clinical outcomes of the patients. NDRG2 and NDRG3 were both positively expressed in the cytoplasm and nucleus of cells in PCa and corresponding noncancerous prostate tissues. The number of positive-staining cells showing immunoreactivity in ten representative microscopic fields was counted and the percentage of positive cells was calculated. The percentage scoring of immunoreactive tumor cells was as follows: 0 (0%), 1 (1-10%), 2 (11-50%) and 3 (>50%). The staining intensity was visually scored and stratified as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). A final immunoreactivity score (IRS) was obtained for each case by multiplying the percentage and the intensity score.

Statistical analysis

The software of SPSS version12.0 for Windows (SPSS Inc, IL, USA) was used for statistical analysis. Continuous variables were expressed as $\bar{x}\pm s$. Statistical analysis was performed with Fisher's exact test for any 2x2 tables, Pearson χ^2 test for non- 2x2 tables, chi-square trend test for ordinal datum, Kaplan-Meier and Cox Regression methods for the question of survival analysis. Differences were considered statistically significant when P was less than 0.05.

Results

Sub-cellular localization and expression patterns of NDRG2 and NDRG3 in PCa tissues

The expression levels of NDRG2 and NDRG3 proteins were detected and analyzed in 20 pairs of fresh primary PCa and corresponding noncancerous prostate tissue samples by Western blot analysis. As shown in Fig. 1, the expression of NDRG2 protein was significantly lower in PCa tissues than those in noncancerous prostate tissues (0.82±0.03 vs. 1.51±0.08, P<0.001), while the expression of NDRG3 protein was

Table 1. Correlation of NDRG2 and NDRG3 expression with clinicopathological features of PCa patients.

Clinicopathological features	Cases No (n, %)	Low NDRG2 expression (n, %)	P	High NDRG3 expression (n, %)	P
Age					
<60	96 (46.60)	59 (61.46)	NS	54 (56.25)	NS
≥60	110 (53.40)	79 (71.82)		72 (65.45)	
PSA at diagnosis					
<10 ng/mL	40 (19.42)	30 (75.00)	NS	26 (65.00)	NS
≥10 ng/mL	166 (80.58)	108 (65.06)		100 (60.24)	
Gleason score					
4-6	62 (30.10)	21 (33.87)	0.003	31 (50.00)	NS
7	92 (44.66)	69 (75.00)		63 (68.48)	
8-10	52 (25.24)	48 (92.31)		32 (61.54)	
Pathological stage					
T1	10 (4.85)	1 (10.00)	0.001	1 (10.00)	0.006
T2	116 (56.31)	65 (56.03)		65 (56.03)	
T3-4	80 (38.83)	72 (90.00)		60 (75.00)	
Metastatic status					
Negative	146 (70.87)	82 (56.16)	0.001	70 (55.56)	0.001
Positive	60 (29.13)	56 (93.33)		56 (93.33)	
Lymph node status					
Negative	140 (67.96)	88 (62.86)	NS	70 (50.00)	0.002
Positive	66 (32.04)	50 (75.76)		56 (84.85)	
Surgical margin status					
Negative	138 (66.99)	96 (69.57)	NS	90 (65.22)	NS
Positive	68 (33.01)	42 (61.76)		36 (52.94)	

Note: 'NS' refers to differences without statistical significance.

significantly higher in PCa tissues than those in noncancerous prostate tissues (1.37±0.06 vs. 0.92±0.03, P<0.001). In addition, the immunohistochemistry analysis was performed to detect the sub-cellular localization and the expression patterns of NDRG2 and NDRG3 proteins in 206 pairs of PCa and corresponding noncancerous prostate tissues. Similar to the results of Western blot analysis, the positive staining of NDRG2 in PCa tissues (Fig. 2A) was dramatically weaker than that in corresponding noncancerous prostate tissues (Fig. 2B). In contrast, NDRG3 immunostainings in PCa tissues (Fig. 2C) were significantly stronger than those in corresponding noncancerous prostate tissues (Fig. 2D). Moreover, the IRS for NDRG2 expression in PCa tissues was significantly lower than that in corresponding noncancerous prostate tissues (mean±S.D.: 2.62±0.89 vs. 5.07±0.58, P<0.001), while the IRS for NDRG3 expression in PCa tissues was significantly higher than that in corresponding noncancerous prostate tissues (mean±S.D.: 6.13±1.07 vs. 2.29±0.82, P<0.001). Moreover, the median of IRS for NDRG2 expression in all PCa tissues was 2.65. Thus, the NDRG2 protein expression levels were further analyzed by classifying IRS values as low (n=138, based on a IRS value less than 2.65) and as high (n=68, based on a IRS value greater than 2.65). The median of IRS for NDRG3 expression in all PCa tissues was 6.10. Thus, the NDRG3 protein expression levels were further analyzed by classifying IRS values as low (n=80, based on a IRS value less than 6.10) and as high (n=126, based

Table 2. Univariate survival analysis of recurrence-free survival (RFS) and overall survival (OS) in 206 patients with PCa.

Variables	RFS			OS		
	RR	95%CI	P	RR	95%CI	P
Age (<60 vs. ≥60)	7.62	0.79-16.93	NS	8.23	0.62-18.56	NS
PSA at diagnosis (<10 ng/mL vs. ≥10 ng/mL)	6.34	0.61-13.08	NS	7.82	0.67-16.32	NS
Gleason score (4-6 vs. 7-10)	9.26	1.01-18.32	NS	12.30	1.22-24.09	0.01
Pathological stage (T1-2 vs. T3-4)	22.61	2.10-45.28	<0.001	25.29	2.30-50.97	<0.001
Metastatic status (Negative vs. Positive)	18.13	1.12-36.86	0.002	15.62	1.10-31.28	0.006
Lymph node status (Negative vs. Positive)	18.62	1.16-37.08	0.002	15.39	1.10-31.22	0.006
Surgical margin status (Negative vs. Positive)	5.61	0.80-12.82	NS	6.29	0.91-15.88	NS
NDRG2 expression (High vs. Low)	20.27	2.29-42.18	<0.001	22.02	2.30-45.69	<0.001
NDRG3 expression (Low vs. High)	19.19	2.03-40.32	<0.001	21.86	2.06-42.96	<0.001

Note: 'NS' refers to differences without statistical significance.

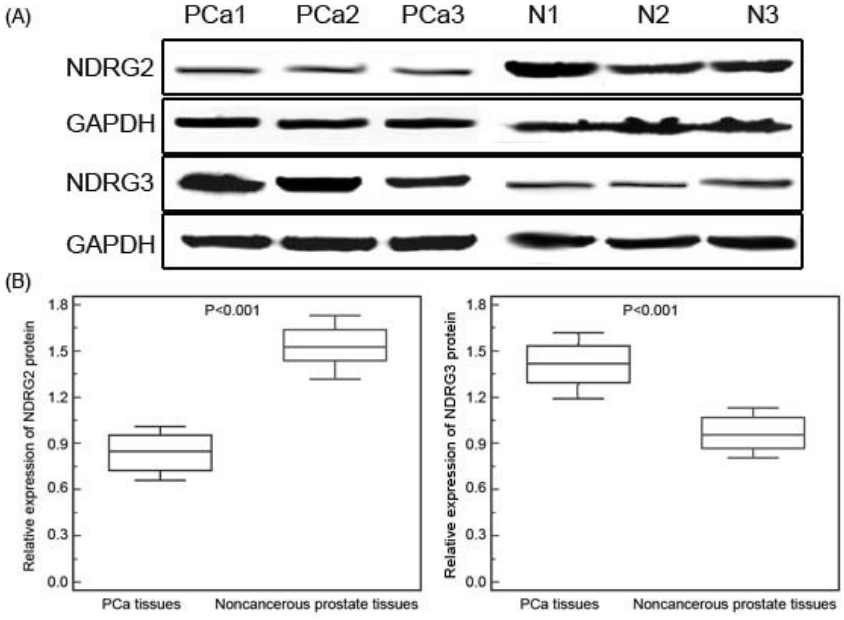

Fig. 1. Expression of NDRG2 and NDRG3 proteins in PCa and noncancerous prostate tissues. **A.** Western blot image for NDRG2 and NDRG3 protein expression in 3 pairs of PCa (PCa1~PCa3) and noncancerous prostate tissues (N1~N3). **B.** Statistical results of NDRG2 and NDRG3 protein expression in 20 pairs of PCa and noncancerous prostate tissues.

on a IRS value greater than 6.10).

Correlation of NDRG2 and NDRG3 expression with the clinicopathological features of PCa patients

To evaluate the association of the two proteins with tumor biology, the clinicopathological features were correlated with NDRG2 and NDRG3 expression. As shown in Table 1, the downregulation of NDRG2 in PCa tissues was significantly correlated with advanced pathological stage (P=0.001), positive metastatic status (P=0.001) and high Gleason score (P=0.003), while the upregulation of NDRG3 in PCa tissues was significantly correlated with advanced pathological stage (P=0.006),

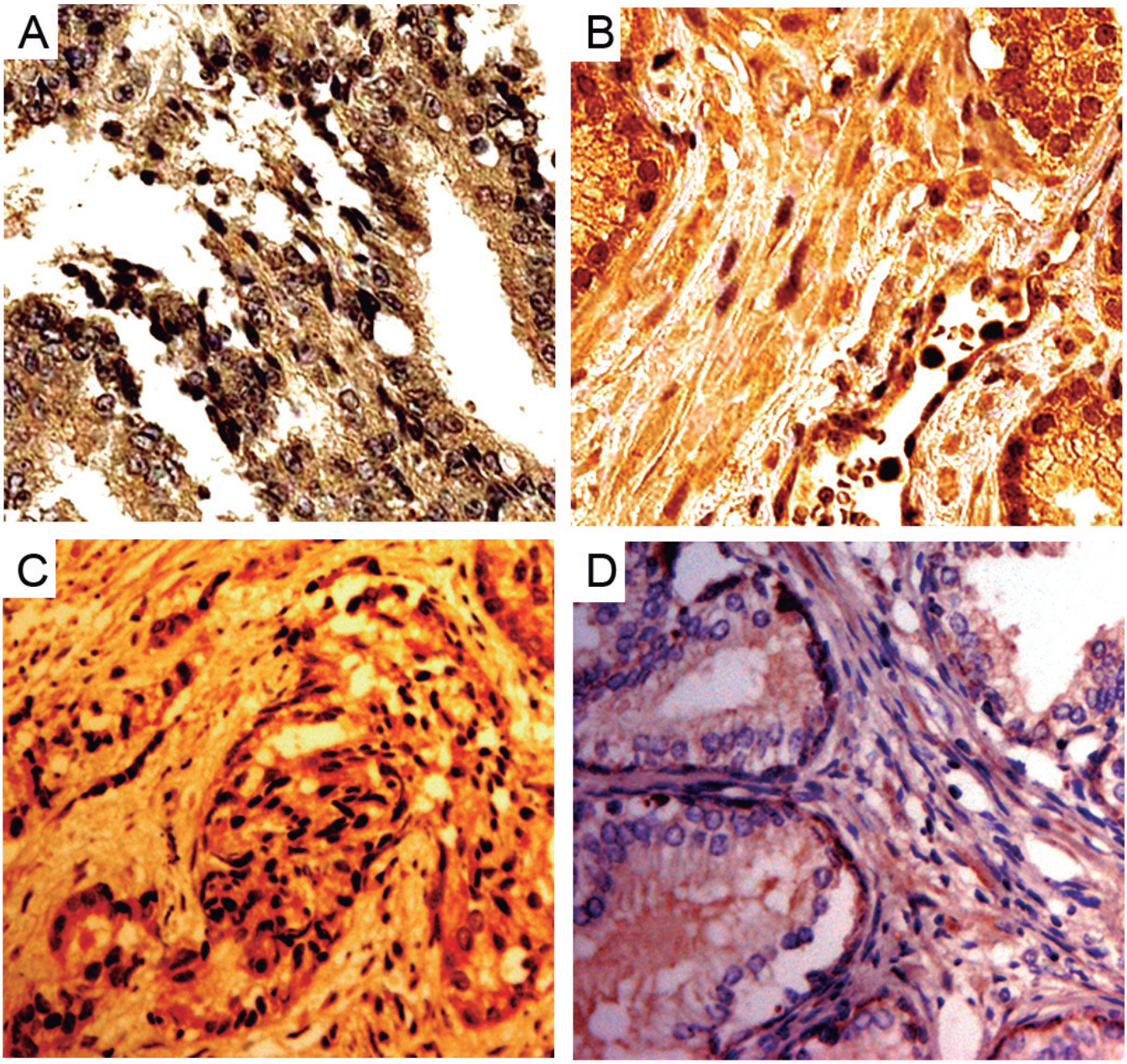

Fig. 2. Immunohistochemical staining for NDRG2 and NDRG3 in PCa tissues. **A.** NDRG2 weakly positive expression was found in cell cytoplasm and nucleus at various levels in PCa tissues. **B.** NDRG2 strongly positive expression was found in cell cytoplasm and nucleus at various levels in noncancerous prostate tissues. **C.** NDRG3 strongly positive expression was found in cell cytoplasm and nucleus at various levels in PCa tissues. **D.** NDRG3 weakly positive expression was found in cell cytoplasm and nucleus at various levels in noncancerous prostate tissues. x 200

Table 3. Multivariate survival analysis of recurrence-free survival (RFS) and overall survival (OS) in 206 patients with PCa.

Variables	RFS			OS		
	RR	95%CI	P	RR	95%CI	P
Gleason score	-	-	-	8.23	1.02-17.56	NS
Pathological stage	18.68	1.10-37.21	0.001	17.93	1.10-35.96	0.001
Metastatic status	12.19	1.01-25.36	0.01	11.68	1.01-23.28	0.02
Lymph node status	12.26	1.02-25.48	0.01	11.19	1.01-23.26	0.02
NDRG2 expression	15.63	1.19-32.87	0.006	15.27	1.13-32.58	0.006
NDRG3 expression	14.02	1.08-30.29	0.008	13.89	1.06-27.62	0.008

Note: 'NS' refers to differences without statistical significance.

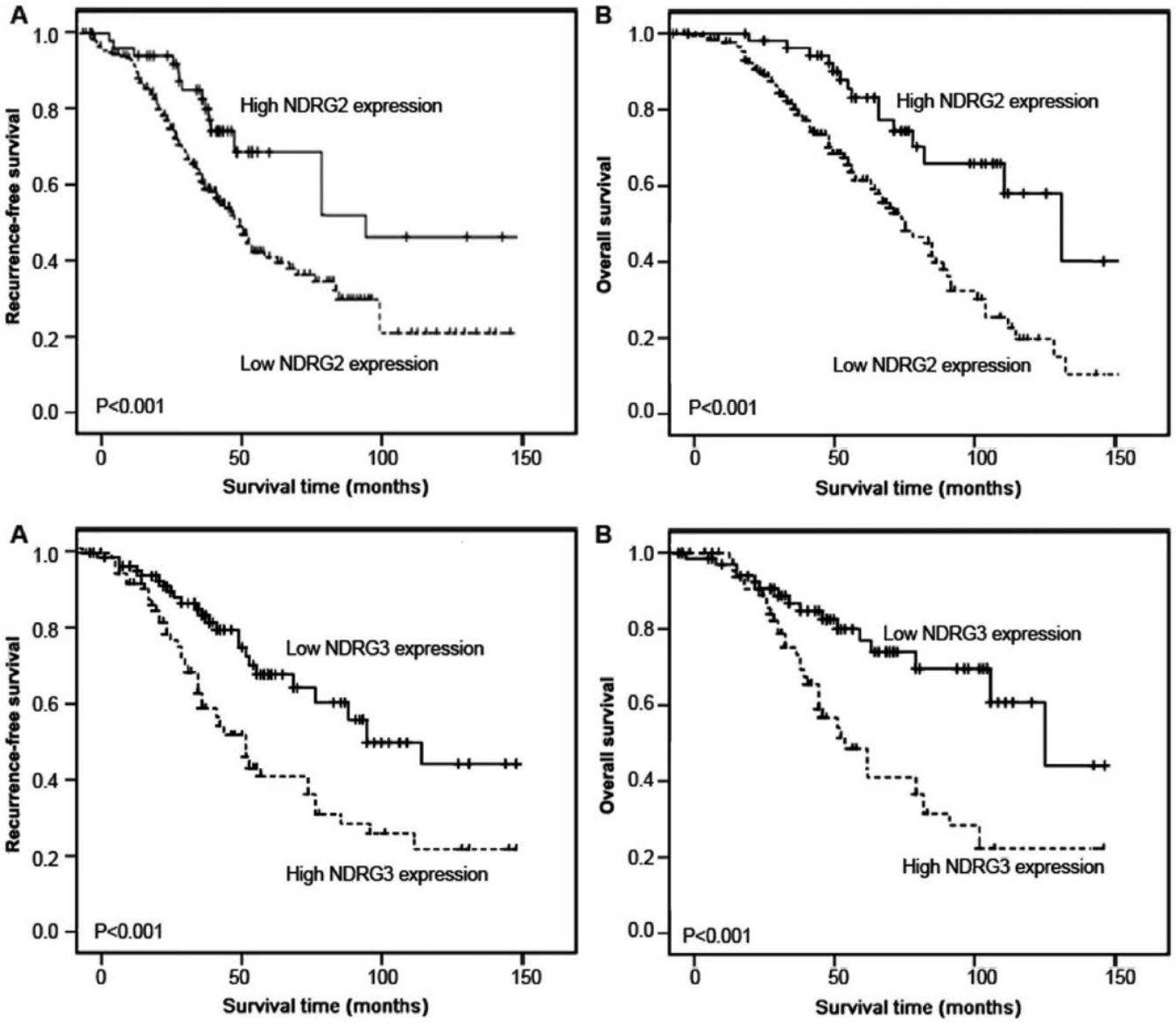

Fig. 3. Recurrence-free survival (RFS) and overall survival (OS) curves for two groups defined by low and high expression of NDRG2 **(A and B)** or NDRG3 **(C and D)** in patients with PCa. The patients with low NDRG2 expression and high NDRG3 expression had a significantly worse OS and RFS than those with high NDRG2 expression and low NDRG3 expression (both P<0.001).

positive metastatic status (P=0.001) and lymph node status (P=0.002).

Prognostic implications of NDRG2 and NDRG3 expression in PCa patients

To assess the possible prognostic values of NDRG2 and NDRG3, we performed PSA-based RFS and OS analysis for all the PCa patients undergoing radical prostatectomy. By univariate analysis, the down-regulation of NDRG2 and the upregulation of NDRG3 were both significantly associated with short RFS (Fig. 3A,B; both P<0.001) and OS (Fig. 3C,D; both P<0.001). Other clinicopathological variables associated with short RFS were advanced pathological stage (P<0.001), positive metastatic status (P=0.002), and positive lymph node status (P=0.002, Table 2). In addition, advanced pathological stage (P<0.001), positive metastatic status (P=0.006), high Gleason score (P=0.01), and positive lymph node status (P=0.006) were all associated with short OS (Table 2).

Furthermore, the Cox multivariate analysis demonstrated the value of NDRG2 and NDRG3 expression and other clinicopathologic features for predicting RFS and OS of PCa patients. As shown in Table 3, pathological stage (both P=0.001), metastatic status (P=0.01 and 0.02, respectively), lymph node status (P=0.01 and 0.02, respectively), NDRG2 expression (both P=0.006) and NDRG3 expression (both P=0.008) were all independent prognostic factors for predicting both RFS and OS of PCa patients.

Discussion

PCa is characterized by rapid progression, easy metastasis, and frequent recurrence. The identification of improved diagnostic and prognostic biomarkers may ensure early detection of aggressive PCa and allow the development of new treatment strategies with less morbidity. In the current study, we detected the aberrant expression of NDRG2 and NDRG3 proteins in a large number of PCa. At first, we found that NDRG2 protein was downregulated and NDRG3 protein was upregulated in PCa tissues compared with corresponding noncancerous prostate tissues. Then, our data showed that the downregulation of NDRG2 and the upregulation of NDRG3 were significantly associated with aggressive tumor progression of PCa. After that, we also observed that the downregulation of NDRG2 and the upregulation of NDRG3 were both associated with short RFS and OS in PCa patients. To the best of our knowledge, this is the first study to reveal the clinical values of NDRG2 and NDRG3 in tumor progression and tumor prognosis of human PCa.

NDRG2, a differentiation-related gene, is one of four members belonging to the NDRG family. Deng et al. (2003) first described the human NDRG2 sequence as a protein containing an acyl-carrier protein (ACP)-like domain. The gene is located on human chromosome 14q11.2 and encodes a 41 kDa protein (Joint Center for Structural Genomics, 2008). Recent studies have demonstrated that NDR2 expression was repressed by c-Myc at transcription level and up-regulated by hypoxia and nickel reagent (Okuda and Kondoh, 1999). Functionally, NDRG2 has been indicated to be involved in cell growth, differentiation, organ formation, and stress injury (Qu et al., 2002). As a gene that is regulated downstream of Myc, NDRG2 expression has been shown to be reduced in many types of human cancers. It is downregulated or lost in various tumors and tumor cell lines, including colorectal, liver and thyroid cancers, as well as glioblastoma (Choi et al., 2007; Liu et al., 2007; Lorentzen et al., 2007). Its overexpression may inhibit tumor cell metastasis and invasion, and may correlate with an improved prognosis in gastric cancer, high-grade glioma and hepatocellular carcinomas (Assämäki et al., 2007; Felsberg et al., 2006; Hu et al., 2004; Hummerich et al., 2006). These findings suggest that NDRG2 might have a role in suppressing carcinogenesis. Regarding human PCa, Yu et al. (Yu et al., 2011) firstly investigated the expression of NDRG2 in PCa tissue and in different PCa cell lines. They found that the expression of the NDRG2 gene was low in PCa cells, and adenovirus-mediated NDRG2 may suppress the proliferation of PC3 cells significantly both *in vitro* and *in vivo*. Then, Gao et al. (2011) further indicated that NDRG2 overexpression may inhibit tumor growth and invasion, and may decrease bone destruction caused by PCa bone metastasis. In line with these previous studies, the current data found the downregulation of NDRG2 in PCa tissues compared with the corresponding non-cancerous prostate tissues. Besides this, our data also showed the significant association between low NDR2 expression and aggressive tumor progression, including advanced pathological stage, positive metastatic status and high Gleason score. More importantly, the downregulation of NDRG2 could independently predict poor RFS and OS in PCa patients.

Another NDRG member is the NDRG3 gene located on human chromosome 20q11.21-11.23 (20q12-11.23) which contains 2588 bp (Zhao et al., 2011). It encodes a 363 amino acid polypeptide (40 kD) highly related to mouse NDRG3 protein (Yang et al., 2013). NDRG3 is highly expressed in many organs including testis, ovary, prostate, spinal cord and thymus rudiment, but the highest expression levels were found in the brain, followed by the heart and kidney (Zhou et al., 2001). Accumulating reports have been indicating the involvement of NDRG3 in human malignancies. Especially in PCa, Wang et al. (Wang et al., 2009) firstly verified the overexpression of NDRG3 in PCa cell lines, which was consistent with our findings in human PCa tissues. In addition to its oncogenic functions for PCa cell lines which was previously reported, we here further found the positive correlation between high NDRG3 expression and aggressive clinicopathological features, including advanced pathological stage, positive metastatic status and lymph node status. Notably, the

upregulation of NDRG3 was also significantly associated with poor RFS and OS in PCa patients, suggesting an important role of NDRG3 in tumor progression and clinical outcome of PCa patients.

In conclusion, our data offer convincing evidence for the first time that the aberrant expression of NDRG2 and NDRG3 may contribute to the malignant progression of PCa. More importantly, both the downregulation of NDRG2 and the upregulation of NDRG3 may be efficient prognostic indicators for PCa.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Accepted November 11, 2013