

Evaluation of discoidin domain receptor-2 (DDR2) expression level in normal, benign, and malignant human prostate tissues

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Abstract

Discoidin domain receptor (DDR) is a new member of the receptor tyrosine kinase family. There are two isoforms of discoidin domain receptor (DDR), DDR1 and DDR2. These receptors play a major role in the adhesion, motility and cell proliferation. Due to the important role of DDR2 in the development of tumor extension, this receptor is pivotal in the field of carcinogenesis. The aim of this study was to investigate the mRNA and protein expression of DDR2, in the malignant, benign prostatic hyperplasia (BPH) and normal tissues of patients with prostate cancer. In this study the gene and protein expression of DDR2 in adjacent normal (n=40), BPH (n=40), and malignant (n=40) prostate tissue were measured using real-time PCR and Western blotting. Then, the correlation of DDR2 gene and protein expression with prognostic factors such as age, tumor grade, tumor stage, lymph node involvement, and serum prostate-specific antigen (PSA) concentration were evaluated. The relative mRNA and protein expression level of DDR2 in malignant and benign prostate tissue was significantly higher than those of adjacent normal tissues ($P < 0.01$). This expression was found to increase approximately 3.5 and 2.1 fold for mRNA and protein levels, respectively. Spearman test indicated a significant correlation between DDR2 mRNA and protein expression with prognostic factors such as tumor grade, stage, lymph node involvement, and serum PSA concentration. However, significant correlation with age was not observed. These findings suggest that DDR2 is a cancer-related gene associated with the aggressive progression of prostate cancer patients.

Keywords: Discoidin domain receptor2 (DDR2); Prostate cancer; Prognostic factors

INTRODUCTION

Prostate cancer is the most common cancer in men. It was also noted that prostate cancer is the second leading cause of cancer death in men (1-3). For this reason, the investigation of the mechanisms that inhibit the growth of prostate cancer cells could be beneficial for the treatment of prostate cancer. Transmission of molecular signals from outside the cell into the cell occurs via receptor-ligand binding and by activation of a cascade of intracellular signaling pathways (4). Inhibition of intracellular signaling pathways could reduce cancer cell proliferation. One of the most important receptors which is involved in

transmitting messages into the cell is receptor tyrosine kinase (RTK) (4). This pathway is involved in cell growth and differentiation in many human cancer cell lines. The RTKs are divided into the 18 sub-family on the basis of the extracellular domain (5). A new member of the RTK family is discoidin domain receptor (DDR) that contains an extracellular domain of 160 amino acids exhibiting strong homology to the dictyostelium discoideum protein discoidin (6). Two isoforms of DDR including DDR1 and DDR2 have been identified (7). DDR1 and DDR2 genes are located in the chromosome 6 (6P21.3) and chromosome 1 (1q23.3) respectively (8). DDR1 and DDR2 are activated by different

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types of collagen and participate in several processes such as cell adhesion, migration, proliferation, and matrix remodeling (9-12). DDR1 is found in highly invasive tumor cells, suggesting its involvement in tumor progression. A number of studies have reported overexpression of DDR2 in lung (13), breast (14), nasopharyngeal cancers (15), suggesting a role for DDR2 in tumor progression. Moreover, the elevated expression of DDR2 in a number of fast-growing invasive tumors suggests that this matrix-activated RTK may be involved in the proliferation and stromal invasion of tumors. The identification of DDRs and the realization that they are unique RTKs promoted investigation of their expression and status in various cancer types, using a variety of approaches. However, several issues should be taken into consideration when evaluating DDRs in cancer tissues. For instance, due to the limitations of the currently available DDR antibodies, immunohistochemical methods cannot provide information on receptor expression of DDR (16).

Analyses of RNA expression by real-time polymerase chain reaction (RT-PCR) or western blotting provide a glimpse on the associations between DDR expression and a variety of cancer-related parameters such as prognostic factors.

The expression of DDR in human prostate cancer tissues has been poorly studied. However, the quantitative mRNA and protein expression of DDR2 in normal, benign prostatic hyperplasia (BPH), and malignant human prostate cancers have not previously been examined and elucidated. Moreover, the correlations between DDR2 expressions and prognostic factors have not yet been characterized in men with prostate cancer.

According to studies on the role of DDR2 in a variety of human cancers, and the involvement of these receptors in tumor growth and progression, the aim of this study was to investigate the mRNA and protein expression of DDR2, in the malignant, BPH, and normal tissues of patients with prostate cancer and to evaluate the relationship between these expressions and some prognostic factors of the disease.

MATERIALS AND METHODS

Material

Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Bologna, Italy). Electrophoresis reagents were prepared from Bio-Rad (Hercules, CA, USA). Glycine, sodium dodecyl sulfate, Tris, polyacrylamide gel electrophoresis (PAGE), N-methylene-bisacrylamide and mercaptoethanol were purchased from Merck Co. (Germany). Rabbit polyclonal antibody against DDR-2 was purchased from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.). Enhanced chemiluminescence (ECL) detection reagent was procured from Amersham (Amersham Corp., Arlington Heights, IL, USA). RNeasy Minikit was supplied by Qiagen (Qiagen Inc, USA). DNase was purchased from Fermentas (Burlington, Ontario, Canada). BCA protein assay kit was obtained from Pierce (TemaRicerca Srl, Bologna, Italy).

Patients and tissue sample preparation

With the local ethical approval and informed consent, 40 patients with prostate cancer, 40 BPH and 40 normal adjacent tissues as a control tissue were included in this study. Tumor tissues were collected during surgery from September 2011 to June 2013 from Cancer Research Center in Imam Khomeini hospital (Iran, Tehran). Normal adjacent tissues were sampled away from the edge of the malignant tumor region. The histopathological diagnosis was confirmed for each sample and pathological tests identifying the type and grade of the cancer were performed. Fresh surgical specimens were frozen immediately and stored in liquid nitrogen. Patients were evaluated on the basis of age, tumor grade, tumor stage, tumor size, prostate-specific antigen (PSA) status, and lymph node involvement (Table 1).

RNA isolation and cDNA synthesis

Total RNA was isolated from the tissues using RNeasy Minikit as described previously (17,18). Briefly, tissues were lysed and homogenized in RLT buffer supplemented with 10 µl/ml mercaptoethanol. The lysate was homogenized with a syringe and a 20-G needle.

Table1. Demographic characteristic of study populations.

	PCa (n=40)	BHP (n=40)	Normal (n=40)
Age (years)	61.3 ± 7.1 (48-70)	53.6 ± 6.3 (42-65)	55.6 ± 7.3 (42-65)
Lymph node status (N)			
N0	22 (55%)		
N1	12 (30%)		
N2	6 (15%)		
Gleason score			
≥7	23 (57.5%)		
<7	17 (42.5%)		
Stage			
II	27 (67.5%)		
III	9 (22.5%)		
IV	4 (10%)		
Tumor size			
≥5 cm	14 (35%)		
<5 cm	26 (65%)		
PSA			
≥10	22 (55%)		
<10	18 (45%)		

PCa; advanced prostate cancer, PSA; prostate-specific antigen.

The sample was added on a silica column followed by washing and eluting in RNase-free water according to the manufacturer's instructions. The RNA concentration was quantified spectrophotometrically at 260 nm and the purity and integrity were determined using the A260/A280 ratio. Total RNA was treated with DNase, and then reverse-transcribed using Revert Aid M-MuLV Reverse Transcriptase with Oligo dT primers according to the manufacturer's protocol.

Semi-quantitative RT-PCR

Quantitative RT-PCR assays of DDR2 (NCBI ID: 4921) cDNA were carried out using gene-specific double fluorescent labeled TaqMan MGB probes (Ce02493471) in a fast RT-PCR system (Applied Bio-systems 7500 fast Real-Time PCR System) according to the manufacturer's instructions. Identical PCR conditions were performed using 1 µl of cDNA, and relative expression levels of genes were normalized to TaqMan probe GAPDH (Hs02758991). The reaction was initiated at 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative mRNA expression level was determined using the $2^{-\Delta\Delta C_t}$ analysis method. This method is

widely used to present relative gene expression (19). These data were then compared between groups and expressed as median fold changes.

Western blot analysis

The DDR2 protein content was detected by western blot analysis. Tissues were pulverized at 4 °C in a lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 0.5 mM phenylmethanesulfonyl fluoride, 100 µM β-glycerol 3-phosphate and 0.5% protease inhibitor cocktail), disrupted by sonication, and centrifuged at 14,000 rpm for 10 min at 4 °C. The protein concentrations of each lysate were determined by BCA protein assay kit. Each protein (30–50 µg) was subjected to sodium dodecyl sulfate-PAGE and transferred on to poly(vinylidene fluoride) membranes. Membranes were incubated with blocking buffer (5% non-fat dry milk in PBS contains 0.1% Tween 20 (PBST)) for 1 h at room temperature. Membranes were then incubated with rabbit polyclonal antibody against DDR-2 overnight at 4 °C, and washed three times (each for 5 min) with PBST. Membranes were incubated with corresponding secondary antibodies for 1 h at room temperature. After washing with PBST, proteins were detected

with ECL detection reagent. The expression of GAPDH was used as an internal standard. The relative amounts of proteins were quantified by densitometry using the ImageJ1.36b software (NIH, Bethesda, MD, USA).

Statistical analysis

Data were presented as mean \pm SD. Mann-Whitney U test and Spearman analysis were used for analysis of the differences among the groups and correlation, respectively. Differences were considered significant when $P < 0.05$. Statistical analysis was performed using Statistical Package for Social Science (SPSS v. 16).

RESULTS

In order to investigate the mRNA and protein expression of DDR2 in prostate cancer, 40 malignant tumors, 40 BPH tissues, and 40 corresponding histologically normal adjacent prostate tissues (Table 1) were analyzed using quantitative RT-PCR and western blotting.

DDR2 mRNA expression

Fig. 1 shows the RT-PCR results of the expression pattern of DDR2. DDR2 mRNA expression in the BPH was found to be higher than those of normal prostate tissues (non-

cancerous). The differences between the means were also found to be statistically significant ($P < 0.05$). As shown in Fig. 1, mean relative DDR2 expression was significantly increased (3.5 fold) in malignant tumors in comparison with those of normal tissue ($P < 0.001$). In addition, mean relative expression in malignant tissue was also significantly ($P < 0.05$) higher than those of BPH patients (Fig. 1).

DDR2 protein expression

To verify the mRNA expression findings, western blotting was performed on prostate malignant tissues, BPH and the normal adjacent tissues to evaluate DDR2 protein expression. The results showed a band for DDR2 at 132 kDa (Fig. 2a), and the protein expression intensity of DDR2 was measured by analyzing the protein bands using densitometry by image-j 1.48 software. We found that DDR2 expression was remarkably increased (2.1 fold) in prostate tumor tissues compared with the corresponding adjacent normal tissues ($P < 0.001$), which was consistent with the quantitative RT-PCR results (Fig. 2b). However, DDR2 protein expression in BPH patients was higher than those of normal tissue, but, the difference of the means was not statistically significant.

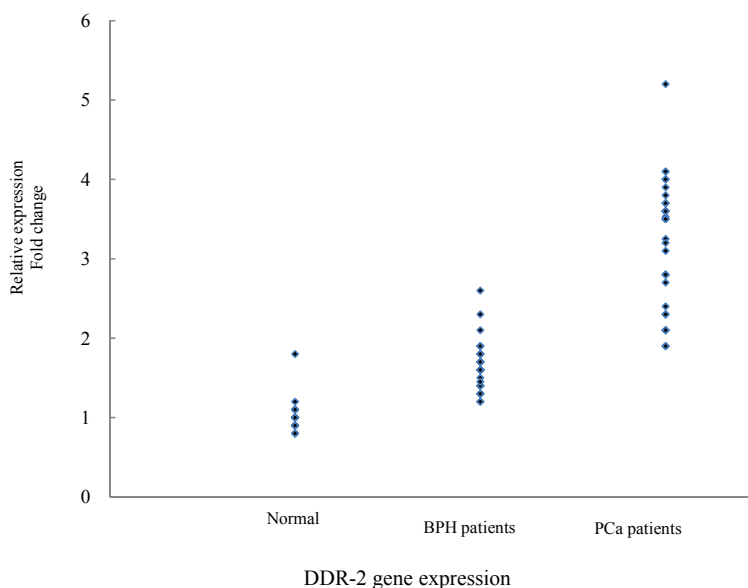


Fig. 1. Relative expression of DDR2 in malignant tissues compared to normal prostate tissues and benign prostatic hyperplasia. * $P < 0.05$, ** $P < 0.01$ are significant.

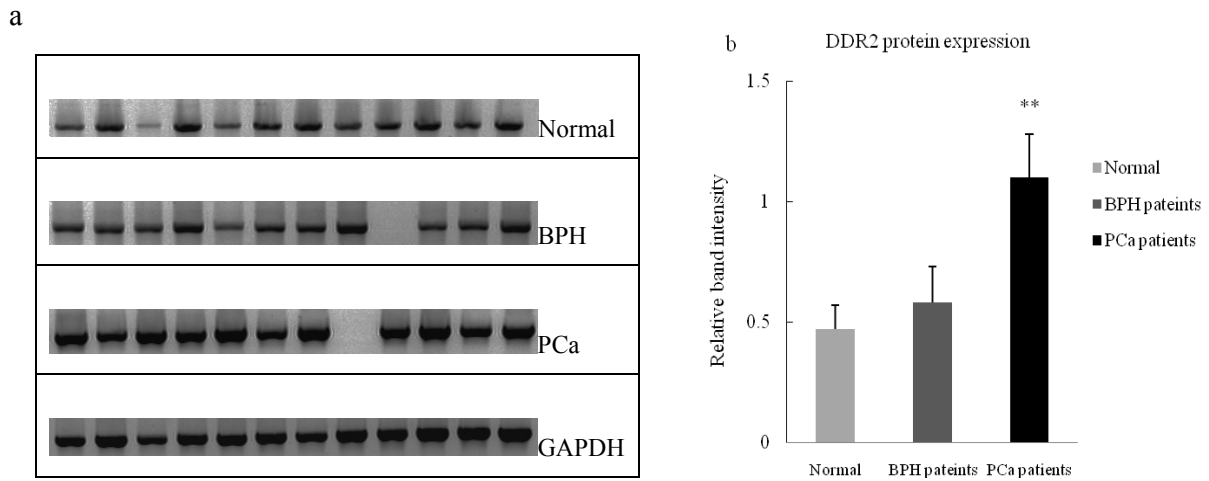


Fig. 2. a; Western blotting detection for DDR2 protein in malignant tissues compared to normal prostate tissues and benign prostatic hyperplasia. b; Protein relative level of DDR2 was calculated by determining intensities of DDR2 bands relative to GAPDH bands using ImageJ software. ** $P < 0.01$ is significant.

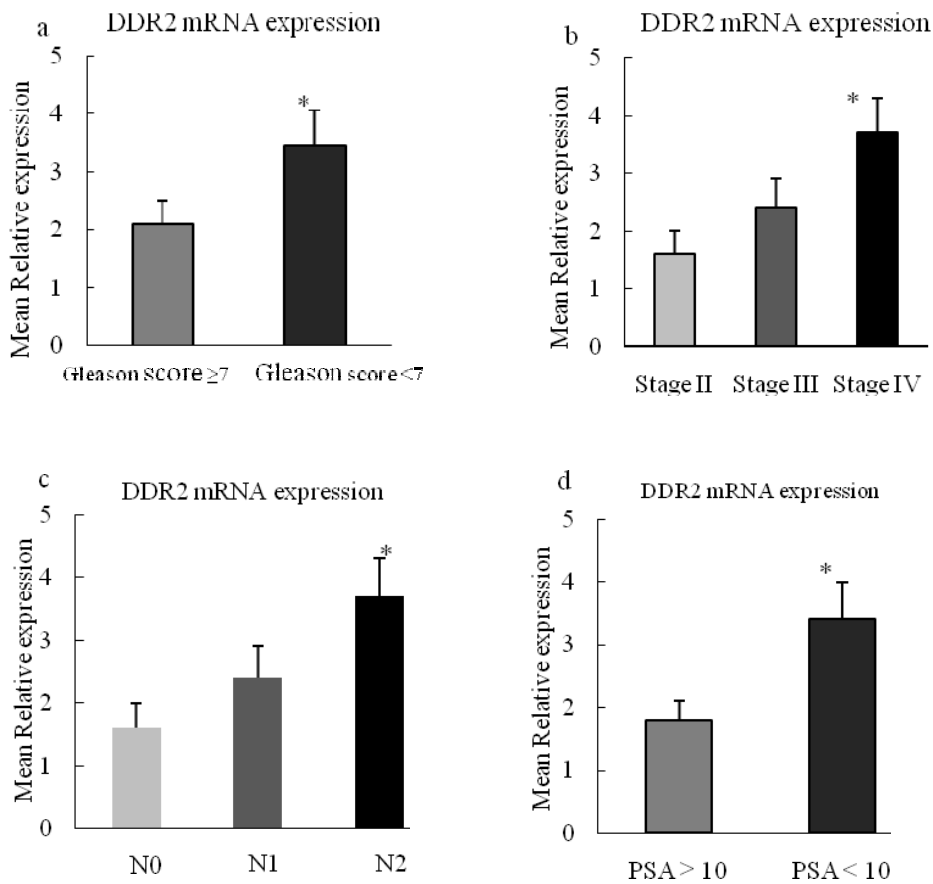


Fig. 3. mRNA relative expression of DDR2 in patients with prostate cancer according to a; tumor grade, b; tumor stage, c; lymph node involvement and d; serum PSA concentration. * $P < 0.05$ is significant.

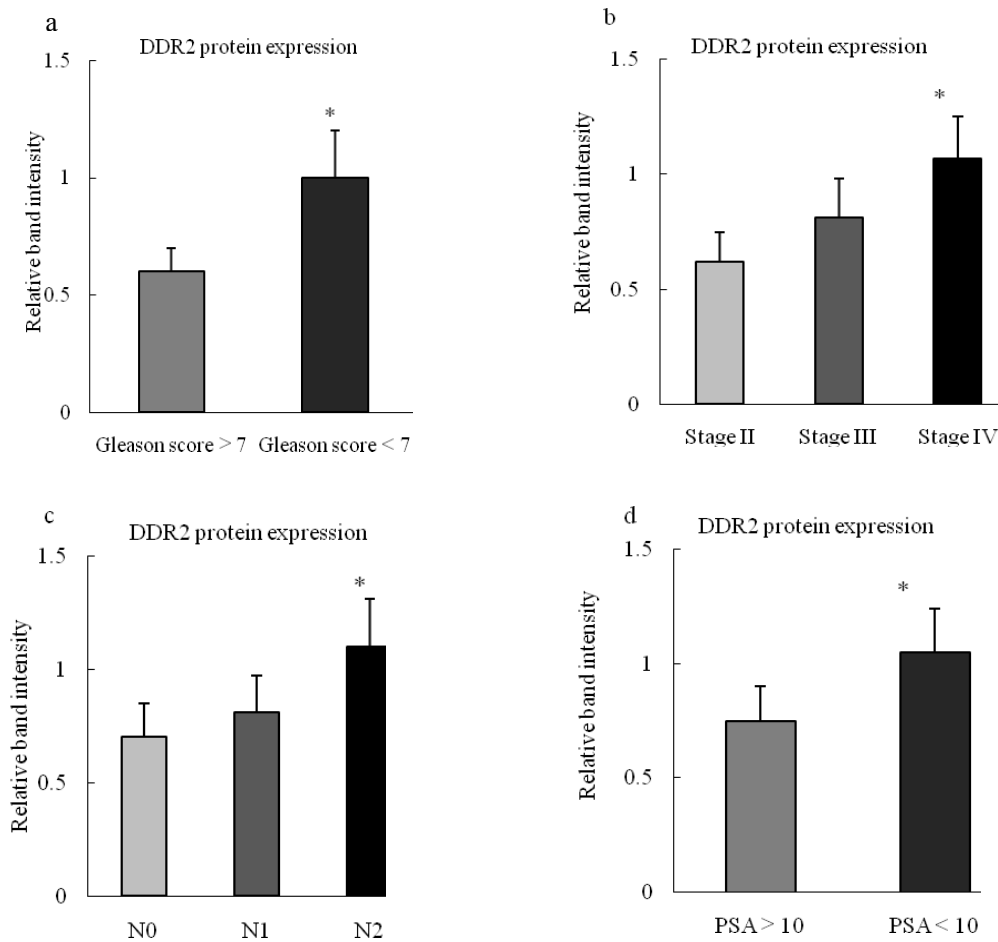


Fig. 4. Protein relative level of DDR2 in patients with prostate cancer according to a; tumor grade, b; tumor stage, c; lymph node involvement and d; serum PSA concentration. * $P < 0.05$ is significant.

Clinical observation

Correlations between DDR2 mRNA, protein expression, and clinicopathological characteristics were analyzed using the Spearman test. A significant positive correlation was found between DDR2 mRNA over-expression and tumor grade ($P < 0.05$), tumor stage ($P < 0.05$), lymph node involvement ($P < 0.05$) and serum PSA concentration ($P < 0.05$) (Fig. 3). Significant positive correlations were also observed between DDR2 protein expression and tumor grade ($P < 0.05$), stage ($P < 0.05$), lymph node involvement ($P < 0.05$) and serum PSA concentration ($P < 0.05$) (Fig. 4). No significant correlation was found between DDR2 mRNA and protein expression and other prognostic factors such as tumor size and age (data not shown).

DISCUSSION

The main findings of the present study are as follows. Firstly, using quantitative RT-PCR assay, DDR2 was identified as genes which were differentially expressed in prostate cancer and BHP compared with non-cancerous prostate tissues. Secondly, the up-regulation of mRNA DDR2 gene in advanced prostate cancer and BPH tissues were further confirmed by protein expression using western blotting analysis. Thirdly, the mRNA and protein expression levels of DDR2 in advanced prostate cancer tissues were related to the prognostic factors.

Recent investigations demonstrated that DDRs have been linked to a number of diverse human cancers. The observations that DDRs can be over-expressed in cancers have

promoted interest in these receptors, particularly as potential therapeutic targets. Analyses of RNA expression by real-time PCR provide a relation between DDR expression and a variety of cancer-related parameters (20). DDR1 and DDR2 were first isolated from a breast cancer cell line and a primary colon adenocarcinoma (7,21). Among the human cancers, most of the advances in DDR research have been made in lung cancer, in particular in non-small cell lung carcinomas. A recent study suggests that DDR2 represents a new therapeutic target in squamous cell carcinoma of the lung (14). Early studies by Ford and coworkers in the 146 primaries NSCLC and in an independent set of 23 matched tumor and normal lung tissue samples showed that DDR2 mRNA was down-regulated (22). Studies in nasopharyngeal carcinomas found high levels of DDR2 mRNA and protein (15). In preliminary study, Shimada and colleagues showed that DDR1 was expressed in human normal and malignant prostate tissues using a qualitative immunohistochemical technique (23). But mRNA or protein expressions of DDR2 have not been elucidated.

In the present study, we identified DDR2 as a differentially expressed in primary epithelial prostate cancer and BPH. DDR2 was more highly expressed in prostate cancer samples compared with normal prostate tissues. We showed that DDR2 expression is associated with prognostic factors such as the tumor grade and stage. Results showed that DDR2 expression was associated with high-grade and advanced stage tumors. These findings were similar to the previous report of Chua and coworkers who demonstrated higher mRNA expression of DDR2 in nasopharyngeal carcinoma (15). Moreover, Microarray analyses in aneuploid papillary thyroid carcinomas revealed that DDR2 is expressed in patients with metastatic disease at time of diagnosis (24). In this study, analysis of tumors from patients demonstrated that DDR2 was over-expressed (24). While these microarray results were validated by quantitative PCR, no further protein expression was performed.

CONCLUSION

In conclusion, our data offer the convincing evidence for the first time that DDR2 mRNA were up-regulated in advanced prostate cancer and BHP tissues. High expression of DDR2 in advanced prostate cancer is strongly correlated with PSA, Gleason score, and lymph node metastasis. These findings suggest that DR2 is a cancer-related gene associated with the aggressive progression of patients with advanced prostate cancer.

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