Original Article

RT-PCR Analysis of ED-A,ED-B, and IIICS Fibronectin Domains: A New Screening Marker For Bladder Cancer

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ABSTRACT

Background: Fibronectin seems to play a very important role in the progression and invasion of bladder cancer. EDA, EDB, and IIICS domains of fibronectin are not expressed in the adult persons but they're expressed in different cancers. The aim of this study is to investigate the mRNA of fibronectin in transitional carcinoma cells (TCC) of bladder to study these domains.

Methods: A total of 20 patients with known bladder cancer were studied. Two of them excluded since their excised tissues were not enough for both the pathological examination and RNA study. Another 20 (control group) were normal volunteers who needed bladder operations. The excised tissue was immediately transferred to RNA*later* (Ambion,TX). RNA was extracted via RNAWIZ (Ambion, TX). cDNA was made via RevertAid First Strand cDNA Synthesis Kit (Fermentas). PCR of the cDNAs was performed using primers for EDA, EDB, and IIICS (Eurogentec,Belgium).

Results: For the first time, we present the expression of the oncofetal fibronectin mRNA in the transitional cell carcinoma of bladder. The high grade muscle invasive (G3T2) tumor, expressed ED-A, ED-B, and IIICS. Expression of ED-A, ED-B, and IIICS was confirmed in the two patients with G3T1 TCC. The four patients with G2Ta and G3Ta expressed both ED-A and ED-B. The four patients with G1T1 tumor expressed ED-A only, similar to the nine patients with G1Ta tumor. None of the normal volunteers expressed the oncofetal extra domains. The sensitivity of ED-A positive fibronectin RNA for detecting TCC of any kind is 100%, and of ED-B was only 35%. The specificity of ED-B positive fibronectin RNA for the high grade TCC is 100%.

Conclusion: ED-A, ED-B, and IIICS could be used as useful markers for the diagnosis and following up of bladder carcinoma.

Keywords*:* Transitional Cell Carcinoma, bladder cancer, fibronectin, RT-PCR, oncofetal.

Bladder cancer is the fourth most common cancer after prostate, lung, and colorectal cancer in men, and is the eighth most common cancer in women[1], and this is the second most common urologic malignancy[2].

Fibronectin is a component of the bladder's extracellular matrix that appears primarily in the basement membrane and submucosa and is absent from the luminal surface of urothelial cells[3].

Fibronectins are dimers of two similar polypeptides that each one is approximately 250 kDa. The multiadhesive property of fibronectin causes its presence in different domains of high-affinity binding sites for collagen and other extracellular matrix (ECM) components and for certain integrins on the surface of cells.

Some researchers measured urinary fibronectin excretion and found it little changed in superficial and superficially invading tumors, but much higher levels were found for deeply invading malignancies. In the last group, 2 to 4 weeks after seeming complete transurethral resection, urinary fibronectin excretion decreased six fold, down to normal levels[4].

A very exciting fact about fibronectin's gene transcription is alternative splicing through which certain exons with introns, are spliced out. Having this phenomenon,different isoforms of fibronectin can be produced[5-7].

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At least 20 different isoforms of fibronectin are known. The exons involved in the process of alternative splicing are named ED-A, ED-B, and IIICS[6,7]. In different cancers, specific combination of these variable exons is believed to produce fairly sensitive and specific fibronectin which are considered as biomarkers or tumor marker. In breast cancer, increased amount of fibronectin was shown to be associated with lymph node involvement and increased mortality[8].

The expression of ED-A, ED-B, and IIICS in adenoid cystic carcinoma of salivary gland produces a different isoform of fibronectin with altered adhesive properties[9]. Fibronectins containing ED-A with or without ED-B segments were approximately twice as potent as those lacking ED-A in their abilities to promote cell adhesion and migration[10]. ED-A and ED-B positive fibronectins has been reported in hepatocellular carcinoma[10]. It's also shown that ED-B positive fibronectin enhances the angiogenesis[9]. Danen et al, also has shown that fibronectin-integrin adhesion stimulates the mitogenic signal transduction pathway. Mitogen activated protein kinase activity is transiently increased by cell attachment to fibronectin resulting in the transcriptional activation of cyclin D1 and p21 genes[11].

Although, there is no report of the expression of extra domain exons (ED-A, ED-B, and IIICS) in bladder carcinomata, most of the research projects in the field of bladder cancer fibronectin, have focused on the quality and quantity of the fibronectin protein, itself, and its diagnostic value as the first approach to and follow up of a bladder cancer patient. What should be emphasized again is the fact that fibronectin's DNA may not be different in various conditions and difference in the characteristics of fibronectin can be attributed to the transcription process (alternative splicing) or post-translational modifications. The latter is mostly responsible for the sugar chain changes seen in this glycoprotein in different situations. Some researchers found a significant higher cellular fibronectin (cFN) plasma levels among patients with TCC of the bladder, compaired with the control group. The differences in cFN plasma levels among pTa/pT1 and/or pT2 stage tumors may indicate a clinically useful potential of this tumor marker for preoperative staging and postoperative follow-up[12].

In a different study oncofetal fibronectin, and not the fibronectin (ordinary plasma and cellular), amount has been assessed in urine of bladder tumor patients. They found a positive result for oncofetal fibronectin in 38 of 40 patients with transitional cell carcinoma of the urinary bladder. Two patients with a small pTaG1-TCC showed negative results. In the urine of healthy controls, no positive results were detected. Thus, there was a sensitivity of 95% and a specificity of 100%[13]. Kirkali et al, showed that fibronectin levels increase in TCC of the human bladder; but there was no correlation between tumor stage, grade, size, multiplicity, and fibronectin levels[14]. This is in contrast to the study of Mutlu et al[15].

The combination of the pathological examination with reverse transcription polymerase chain reaction (RT-PCR) analyses of different oncofetal isoforms of fibronectin can be a useful tool for increasing the sensitivity and specificity of the diagnosis and facilitating, thus, the identification of the more aggressive and invasive bladder carcinoma.

Materials and Methods

In this study, a total of 20 patients with known bladder cancer or first time cystoscopy whom were revealed to have tumoral involvement of the bladder were studied (Table1). Two of them excluded from the study, since the excised tissues were not enough for both pathological examination and RNA study.

Another 20 (control group) were normal volunteers who needed bladder operations(Table2). Among them, there were eighteen men with benign prostatic hyperplasia whom needed open transvesical prostatectomy and two women with vesicovaginal fistula after abdominal hysterectomy for benign conditions. Cystoscopy performed preliminarily, didn't show any suspicious bladder mass in the control group. Informed consent typed in Farsi and English, were taken from both groups.

All the patients underwent general anesthesia. Using störz resectoscope, transurethral resection of the bladder tumor performed. Sizable segments stored in formalin solution for subsequent pathological examination. The excised tissue for RNA study was immediately transferred to a 1.5 cc RNase free eppendorf tube and 10X RNA*later* (Ambion,TX) was added to the tube. A case, in whom radical cystectomy was performed, yielded more

Table1. Distribution of the case and control groups (NS, not significant).

	Mean age (Y) (NS)	Male (n)	Female (n)	Prostatectomy	Vesicovaginal fistula	TURBT	Radical cystectomy
Case	60.3	16	2	-	-	17	1
Control	63.1	18	2	18	2	-	-

bulky specimen; 7 cc RNase free universal tube was ready to contain the bulky specimen.

According to the manufacturer's instruction, the tissues were held in RNAlater at 4° C for 24 hours and then kept in -20° C indefinitely, until the time of RNA extraction. RNA was extracted via RNA_{WIZ} (Ambion, TX) at the department of Genetics and Molecular Biology, Isfahan university of medical sciences. Tissues were snaped frozen in liquid nitrogen immediately after bringing out from the freezer and grinded. One mL of RNA_{WIZ} was added to each hundred mg of the tissue for the subsequent extraction. Finally, the RNAs dried partially by speed vacuum for 3 minutes (concentrator 5301, Eppendorf).

Table 2. Expression of ED-A, ED-B, and IIICS in TCC of bladder according to grade and stage.

	G1Ta	G1T1	G2Ta	G3Ta	G3T1	G3T2
No.	7	4	3	1	2	1
ED-A	7	4	3	1	2	1
ED-B	0	0	3	1	2	1
IIICS	0	0	0	0	2	1

Before freezing the RNAs, spectrophotometry (biophotometer,Eppendorf) of the purified RNAs at 260 and 280 A° was performed and the ratio 260/280 A° and the concentration of the RNA were measured. All the RNAs had ratio between 1.65-2 .

For the controls, about 200 mg of the bladder epithelium was removed using the electrocautery in the line of incision to the bladder which was performed for the therapeutic purposes.

cDNA was made via RevertAid First Strand cDNA Synthesis Kit (Fermentas). Between 1-4 µg of RNA was used to make the cDNA. Oligo(dt) primers were used for the cDNA synthesis. 10 µL of RNA and 0.5 µg of oligo(dt) primer added to each tube and heated at 70° C for 5 minutes. The tubes immediately transferred on ice and 4 µL of 5X reaction buffer, 2 µL of 10mM dNTP mix, 20 units of ribonuclease inhibitor, and 1 µL of deionised water added to each tube (total 19 µL). The mixture heated at 37 ° C for 5 minutes. 200 units of RevertAid M-MuLV reverse transcriptase added to each tube and heated for 60 minutes at 42° C. Reaction was stopped by heating the tubes at 70° C for 10 minutes and soon chilled on ice.

PCR Analyses

Master mixtures made by mixing 80 ng of forward and reverse primers for ED-A, ED-B, and IIICS (each primer pair were added to a master mix tube), 5 µL of 5X PCR buffer, 1 µL of 1mM dNTP, 35 µL of water and, 1 µL of Taq DNA polymerase (total 50 µL).

The primers designed via the software to contain 20 bases, each one (Eurogentec,Belgium). The sequences are as follow:

ED-A forward: 5'-ccaggtacagggtgacctac-3'
ED-A reverse: 5'-ctctccatatcatcgtgcaa-3'
ED-B forward: 5'-cgctaaactcttccaccatt-3'
ED-B reverse: 5'-ccgccattaatgagagtgat-3'
IIICS forward: 5'-ccagagatcttggatgttcc-3'
IIICS reverse: 5'-gcctaaaaccatgttcctca-3'

The Haibaid thermocycler was programmed for 22 amplification cycles according to Toyoshima et al[9].

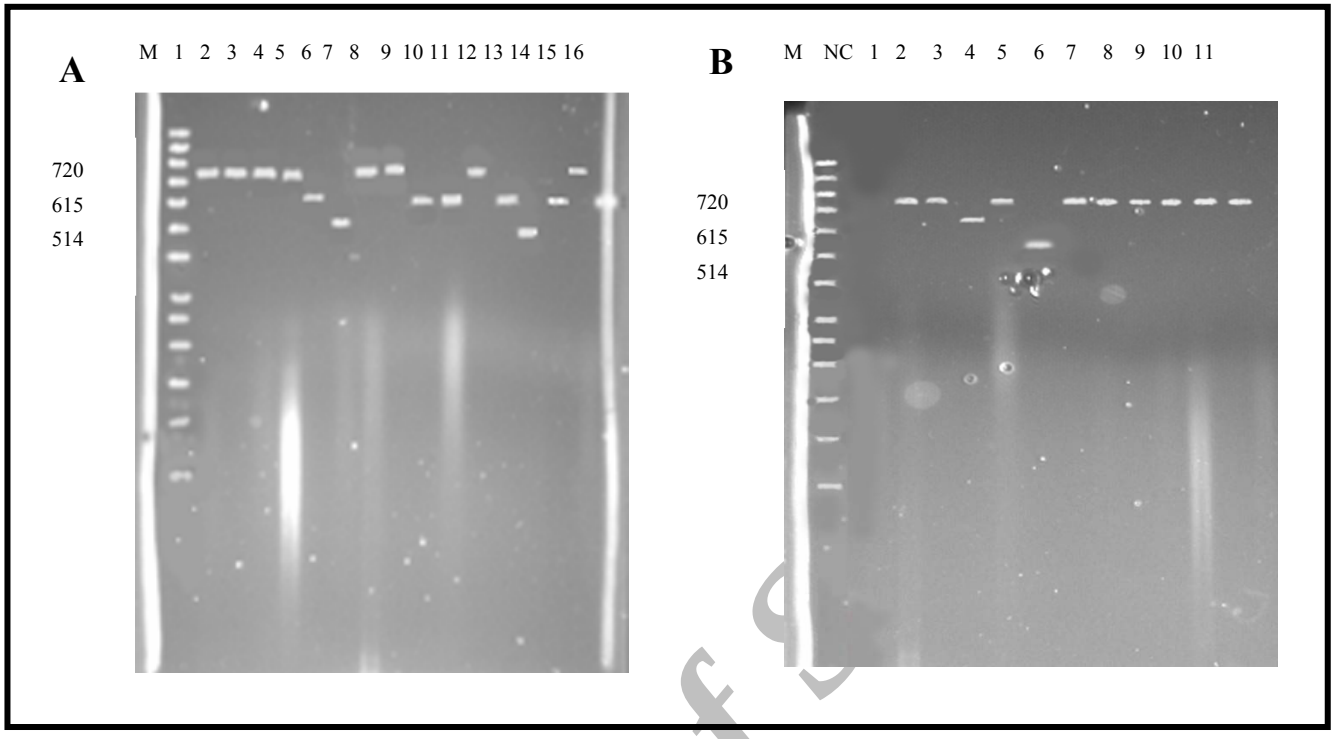

Figure 1. RT-PCR results of bladder TCC fibronectin. The electrophoresis performed on 2% agarose gel. Gel A demonstrates ED-A in columns 1-4,7,8,11, and 15 (720 b); ED-B in columns 5,9,10,12,14, and 16 (615 b); and IIICS in columns 6 and 13 (514 b). Gel B demonstrates ED-A in columns 1, 2, 4, and 6-11. It also demonstrates ED-B and IIICS in columns 3 and 5 respectively. M, DNA size marker NC, negative control.

The amplified DNA segments were analysed by electrophoresis on 2% agarose gels, using O'GeneRuler 50bp DNA Ladder as DNA size marker.

Results

The PCR products of the 18 TCC tumors loaded into 2% agarose gel. The electrophoresis performed at 100 V for 80 minutes (figure 1).

None of the normal volunteers expressed the oncofetal extra domains (data not shown). Table2 shows the expression of the oncofetal domains of the fibronectin in different stages and grades. The high grade muscle invasive TCC of the bladder expressed ED-A, ED-B, and IIICS. This tumor was retrieved through an extensive procedure, i.e. radical cystectomy. A patient suffered recurrent superficial high grade tumor and he was a heavy smoker who couldn't quit even in the preoperative period. Expression of ED-A, ED-B, and IIICS was confirmed in the high grade TCC invading lamina propria (G3T1). The high grade tumor confined to epithelium (G2Ta and G3Ta) expressed both ED-A and ED-B. The G1T1 tumors expressed ED-A only. None of these tumors expressed either of ED-B or IIICS. Papillary superficial low grade tumors expressed only ED-A domain.

Discussion

A very exiting fact about fibronectin's gene transcription is alternative splicing through which certain exons with introns are spliced out. Having this phenomenon, different isoforms of fibronectin can be produced[5-7].

At least, 20 different isoforms of fibronectin are known. The extra domains, produced in the process of alternative splicing are named ED-A, ED-B, and IIICS[6,7]. In different cancers, combination of these variable exons, produce fairly sensitive and specific fibronectins, accordingly, to be considered as biomarkers, i.e. Tumor markers.

Fibronectins containing ED-A with or without ED-B segments were approximately twice as potent as those lacking ED-A in their abilities to promote cell adhesion and migration[16].

ED-A and ED-B positive fibronectins have been reported in hepatocellular carcinoma.It's also shown that ED-B positive fibronectin enhances the angiogenesis[9].

It is assumed that extracellular matrix elements dramatically affect growth, multiplication, and differentiation of cells in culture, and the proficiency of cells to grow, fairly independently from cell-matrix interactions is a key event in malignant transformation[17-19].

As well as the fact that ED-A, ED-B, and IIICS not expressed in the adult persons – so called oncofetal fibronectins- are considered as potential bladder carcinoma tumor markers to be checked for in the follow up period of TCC or even estimating the stage and/or grade of the tumor, inhibition of the adhesion of the malignant cells of the transitional epithelium to the Extracellular matrix components, specially the oncofetal fibronectin could theoretically and practically hamper the spreading and local invasion of the neoplastic cells[20-22].

The present study confirms, for the first time, the expression of oncofetal fibronectin mRNA in the transitional cell carcinoma of bladder. The expression of IIICS extra domain of the fibronectin mRNA was limited to the high grade muscle invasive TCC. The low grade superficial TCC of bladder only expressed ED-A.

The RT-PCR identification of bladder tumors seems to be a highly sensitive method to diagnose bladder transitional cell carcinoma, as the sensitivity of ED-A positive fibronectin RNA for detecting TCC of any kind is 100%. The specificity of IIICS positive fibronectin RNA for detecting the muscle invasive high grade TCC is again 100%. The sensitivity of ED-B positive fibronectin RNA for detecting TCC of bladder is only 35%. The specificity of ED-B positive fibronectin RNA for the high grade TCC is 100%. It means that finding the ED-B and/or IIICS positive fibronectin RNA predicts a higher grade and, therefore, portends a poorer prognosis. It seems also applicable to perform RT-PCR investigation of fibronectin's RNA in the follow up period of the patients with bladder TCCs, and looking for the expression of oncofetal fibronectin isoforms.

Finally, any doubt about the neoplastic nature of a bladder mass lesion, warrants RT-PCR analysis of the fibronectin RNA. The expression of ED-A positive fibronectin RNA suggests the presence of a neoplastic lesion, and identification of ED-B and/or IIICS recommend a closer follow up protocol, not to overlook a high grade and invasive bladder carcinoma and to increase the life expectancy of the patient.

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