NY-ESO-1 expression and immunogenicity in prostate cancer patients

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Etude de l'expression et de l'immunogénécité de NY-ESO-1 chez les patients atteints du cancer de la prostate

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RÉSUMÉ

Prérequis: Le cancer de la prostate est responsable d'une morbidité importante. Le traitement de référence des formes localement avancées et métastatiques est l'hormonothérapie. Cependant, après un phénomène d'échappement hormonal, l'immunothérapie constitue une alternative de choix.

But : Etudier l'expression de l'antigène NY-ESO-1 par les tumeurs prostatiques et mieux caractériser son immunogénicité.

Méthodes : L'étude a porté sur 23 patients ayant un adénocarcinome et 23 patients atteints de tumeurs bénignes de la prostate. L'expression de l'antigène Cancer Testis NY-ESO-1 a été étudiée par RT-PCR, via une paire d'amorce spécifique. Par ailleurs, la réponse immune humorale spontanée dirigée contre l'antigène NY-ESO-1 a été étudiée par ELISA.

Résultats : Nos résultats montrent que l'antigène NY-ESO-1 est exprimé chez 39% (9/23) des patients atteints d'un cancer de la prostate. Par ailleurs, la présence d'anticorps spécifiques a été détectée dans 52,57 %(12/23) des cas dont 55%(5/9) exprimant l'antigène NY-ESO-1. En revanche, aucune expression de NY-ESO-1 ni d'anticorps spécifiques à cet antigène n'a été retrouvée chez les patients atteints de tumeurs bénignes de la prostate.

Conclusion : L'ensemble de ces résultats montre l'expression de l'antigène NY-ESO-1 par les tumeurs prostatiques et son immunogénicité, avançant le rôle de cette protéine dans le diagnostic, l'estimation du pronostic et son utilisation dans d'éventuelles immunothérapies anti-cancéreuse chez les patients atteints d'un adénocarcinome prostatique.

Mots-clés

Antigène de la famille Cancer Testis ; NY-ESO-1 ; Immunothérapie; Cancer de la prostate

SUMMARY

Background: Prostate cancer is the second leading cause of men cancer-related death. Cancer immunotherapy has been investigated as a treatment which might be instituted at the point of detection of androgen-independent metastatic disease.

Aim: to investigate the expression and humoral response against NY-ESO-1 in patients with prostate cancer (PC) and to analyze the relationship between expression of NY-ESO-1 and clinicopathological features.

Methods: NY-ESO-1 mRNA in surgically resected PC and benign prostatic hyperplasia (BPH) were examined by reverse transcription-polymerase chain reaction. The antibody response to NY-ESO-1 was examined by enzyme-linked Elisa assay using recombinant NY-ESO-1 protein.

Results: NY-ESO-1 mRNA was detected in 9 of 23 (39%) PC patients. Antibodies against NY-ESO-1 protein were detected in 12 of 23 (52%) sera of PC patients and in 5 of 9 (55%) of NY-ESO-1 expressing tumors. However, no mRNA copy or NY-ESO-1 antibodies were detected in all BPH patients tested.

Conclusion : The present study has demonstrated the expression of NY-ESO-1mRNA in prostate Cancer patients and NY-ESO-1 antibody production. Our data suggest that NY-ESO-1 could be used as a tumor marker and constitute a good candidate for vaccine-based immunotherapy for hormonal resistant prostate cancer patients.

Key-words

Cancer testis antigen- Immunotherapy- NY-ESO-1- Prostate cancer

Prostate cancer (PC) is the second leading cause of cancer related death in men [1]. Although the advent of serum prostate-specific antigen (PSA) testing has increased significantly early detection of PC, the number of deaths from PC has only decreased slightly. According to the stage of the disease, therapeutic options are different. Effective treatment options include surgery and radiation therapy for patients with localized disease. Whereas, for recurrent and metastatic PC patients, hormone therapy is used as the first line of treatment to which initially patients respond very well. However, most patients fail the therapy and disease progression occurs after few years [2]. Several alternative therapeutic strategies are being actively pursued in order to improve the poor prognosis of these patients. Recently, reports of the clinical efficacy of immunotherapy have generated new hope. Activation of the immune system against tumor cells has led to distinct tumor regression, particularly in patients with melanoma and renal cell carcinoma [3, 4]. Actually, there are only few clinical trials of immunotherapy for metastatic PC. However, cancer vaccine may represent an effective therapeutic strategy for PC patients. The discovery of defined tumor associated antigens (TAA) that induce spontaneous immune responses in different type of human cancer provides several targets for the construction of cancer vaccines [5]. Among the TAA, Cancer Testis antigens (CTA) have become a matter of great interest because of their highly restricted expression to tumor and immunogenicity. NY-ESO-1 is one of the most immunogenic CTA, eliciting both humoral and cellular immune responses in patients with NY-ESO-1 expressing tumor [6]. NY-ESO-1 is expressed in a variety of cancer [7] including breast [8], ovarian [9] hepatocarcinoma [10], non-small cell lung cancer [11] and esophageal cancer [12]. It has been estimated that 10% - 50% of advanced NY-ESO-1 positive tumors develop antibody responses [13, 14] coordinated in more than 90% with NY-ESO-1 specific CD8 lymphocyte response [6]. Therefore, NY-ESO-1 expressing PC tumors may be an optimal target for anti-NY-ESO-1 immunotherapy.

In the present study, we examined the frequency of expression of NY-ESO-1 mRNA in PC and BPH patients. We also analysed NY-ESO-1 immunogenicity by investigating the presence of NY-ESO-1 antibodies in the serum from PC patients by enzyme linked immunosorbent assay. These results were correlated to the clinicopathological features in order to evaluate the clinic relevance of NY-ESO-1 in prostate cancer.

MATERIALS AND METHODS

Patients, sera and tumor specimens

A total of 23 newly diagnosed PC tissues and 23 pathologically proven BPH samples from transurethral resection were obtained from the division of urology, Charles Nicolle Hospital, Tunisia. Our routine strategy to diagnose PC included prostate-specific antigen (PSA) serum level and hard nodular lesions palpable on digital rectal examination. All tumors were graded according to the general rule for Clinical and Pathologic Studies on PC. The clinicopathologic characteristics of PC and BPH patients are summarized in Table 1. The fresh tumor tissues were immediately, after surgical removal, minced with scissors and kept at -80°C until RNA extraction. Sera were taken from PC patients before surgical resection and stored at -80°C until use. This study was conducted under approval of Institutional Review Board.

Table 1 : Clinical characteristics of prostate cancer patients and BPH.

Prostate Cancer (PC)	
Total number of patients	23
Age (years)	
Mean	75
Range	60-91
T stage	
T1	6
T2	1
T3	12
T4	4
Gleason score	
<7	9
7	4
>7	10
Preoperative PSA	
<4	1
4-10	3
>10	16
NI	3
Begnin Prostatic hyperplasia (BPH)	
Total number of patients	23
Age (years)	
Mean	70
Range	69-79

Values are number of patients except age in year.

RNA Extraction

Total cellular RNA was extracted from frozen tissue specimens using guanidium-isothiocyanate for denaturation followed by an acidic phenol extraction and isopropanol precipitation. Firstly, the frozen tissue samples are crashed in a mortar filled with liquid nitrogen. The obtained powder was homogenized in 1ml of TRizol Reagent (Invitrogen, US, CA) and incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. After adding 200μl of chloroform (SIGMA, Germany), tubes were incubated at room temperature for 2 to 3 minutes and than centrifuged at 12000g for 15 minutes. Following centrifugation, the mixture separates into lower red phenol chloroform phase, an interphase and a colourless upper aqueous phase. RNA, which remains exclusively in the upper phase, was removed by aspiration. Secondly, RNA is precipated from the aqueous phase by mixing with isopropyl alcohol (500μl per 1ml of TRizol). Samples were incubated for 10 minutes and centrifuged for 15 minutes at 4°C. The RNA precipated forms a visible pellet on the tube side. After removing the supernatant, the RNA pellets were washed once with 75% ice-cold ethanol. Samples are mixed by vortexing and centrifuged at 7500 g for 5 minutes at 4°C. Finally, the RNA pellets were dried for 10 minutes and

resuspended in diethyl pyrocarbonate treated water (DEPC). After incubation for 10 minutes at 55°C, RNA samples were stored at -80C° until used. Total RNA quality and quantity were assessed by absorbency at 260nm using a nucleic acid and protein analyser.

Expression of NY-ESO-1 gene in PC primary tumors by reverse transcription-PCR

Five mg of RNA were primed with an oligo(dT)18 oligonucleotide (0,5μg) by a pre-incubation at 65°C for 5 min to ensure optimal annealing . The primed RNA was reverse-transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogene, US) in the presence of 1,5 μl of 5X First Strand Buffer (Invitrogene, US) [250mM Tris-HCL; 375mM KCL; 15Mm $MgCL_2$], 10 units RNasin (Invitrogen, US) and 1,25 μl of 100 mM DTT, 1,25 μl of each desoxynucleotide triphosphates (Promega, US) at 10 mM in a total volume of 25 μl, incubated at 37°C for 60 min and then cooled at -20°C. The quality of cDNA samples was tested by amplifying NY-ESO-1 and ß-actin as follows: conventional PCR was performed in a 50 μl reaction mixture containing 3 μl of cDNA, 0.7 μl of each deoxynucleotide triphosphates (Promega) at 10Mm, 10X PCR Buffer [100Mm Tris-HCL; 500mM KCL; 15Mm $MgCL_2$], 20 pmol of each primer, and 1.5 unit of Taq DNA Polymerase (GO Taq Promega, UK). After incubation for 10 minutes at 95°C, 35 PCR cycles were run according to the following cycle profile: denaturation at 95°C for 1 min, annealing at Tm°C for 1 minute, and extension at 72°C for 1 minute with a terminal extension at 72°C for 10 minutes. PCR products were then electrophoresed on 1% agarose gels, visualized by ethidium bromide staining (0.2μl/ml) and photographed under ultraviolet light (Gel doc system 2000; Bio Rad). Primer sequences, annealing temperatures and product sizes are described in Table 2. To ensure the specificity of the result, RNA from Melanoma cell line known to express NY-ESO-1 was included as a positive control. To test for the contamination, each PCR reaction series was made in the absence of cDNA.

Table 2 : Primer sequences, annealing temperatures and product size for PCR amplification.

Genes	Primer sequence	Tm°C	
ß actine			**Product**
5' Fw	GGC ATC GTG ATG GACT CCG	62	**size (pb)**
3'Rw	GCTGGAAGG TGG ACA GCG A		
			615
NY-ESO-1			
5' Fw	CAG GGC TGA ATG GAT GCT GCA GA	60	
3'Rw	GCG CCT CTG CCC TGA GGG AGG		
			331

Enzyme-linked immunoabsorbant assay

96-well plates were coated, in a triplicate assay, with 50 ng of recombinant NY-ESO-1 protein (kindly given by Dr A.Zippelius) or with a control protein (SSX2) after dilution in PBST (PBS /0,5% Tween 20). Plates were then incubated overnight at 4°C, consecutively blocked with PBST / 5% FCS for 2 hours at 4°C, and then washed twice. Serum samples diluted 1:250 in PBST / 5% FCS were incubated for 2 hours at room temperature, followed by incubation for 30 min with a secondary, peroxidase-conjugated, goat-anti human-IgG antibody (Sigma, Darmstadt, Germany), diluted 1:20000 in PBST / 5% FCS. Consecutively, the plates were subsequently developed at room temperature for 30 minutes with 150 μl / well TMB (Tetramethylbenzidine Sigma, Darmstadt, Germany) and analyzed using an ELISA reader (Labsystems Multiskan MS) at l=450 nm .

Statistical analysis

Statistical analysis was performed with Fisher's exact probability test. A *P*-value of less than 0.05 was considered significant.

RESULTS

NY-ESO-1 expression in PC and benign prostatic hyperplasia

The expression of NY-ESO-1 mRNA was investigated by RT-PCR in 23 patients and 23 BPH. NY-ESO-1 mRNA was detected in 9 of 23 (39%) of cancer specimens while no expression was observed in patients with benign lesions.

The size of the PCR product from PC was the same as that from NY-ESO-1 positive melanoma cell line and the PCR product was confirmed as NY-ESO-1 by nucleotide sequencing (data non shown). Table 3 summarizes the relationship between NY-ESO-1 mRNA expression and various clinicopathological features. Higher frequency of NY-ESO-1 mRNA expression was observed in stages T3 and T4 (7 of 16, 44%) than in stages T1 and T2 (2 of 7, 28.5%). However, the difference between stage T1/T2 and T3/T4 was not statistically significant. No correlation was observed between NY-ESO-1 mRNA expression and Gleason Score (Table 3), age and PSA levels (data not shown).

Table 3 : Relationship between NY-ESO-1 mRNA expression and NY-ESO-1 antibody response with clinical features

	mRNA positive tumors examined	**p****	**Antiboy positive sera tested (%)**	**p****
All tumor specimens	**9/23 (40%)**		**12/23 (52,1%)**	
Pathological Stage				
T1+T2	2/7 (28,5%)		5/7 (71,4%)	0,405
T3+T4	7/16 (43,7%)	0,52	8/16 (50%)	
Gleason Score				
<7	4/9 (44,4%)		7/14 (50%)	1
≥7	5/14 (36%)	1,4	4/9 (44,4%)	

** $\chi 2$ test for independance

PC patients frequently develop humoral immune responses against NY-ESO-1

Considering the expression of NY-ESO-1 in our series, we analysed the existence of a specific IgG antibody responses against NY-ESO-1 in PC patients. We screened 23 sera derived from PC patients, 23 sera from patients with BPH and 5 sera from healthy individuals, for NY-ESO-1 specific antibody responses by ELISA assays. Using ELISA assay using sera from healthy individuals has allowed the definition of a cut-off value, defined as the mean OD plus 3 x standard deviations. Our results showed that NY-ESO-1 specific antibodies were detected in 12 out of 23 sera (52.1%) derived from PC patients. While, all BPH patients were tested negative. Some positive sera in ELISA assays were confirmed by Western Blotting using the recombinant protein (data not shown). NY-ESO-1 antibody responses were detected in localized and advanced PC: 5 of 7 (71.4%) in T1-T2 and 8 of 16 (50%) in T3-T4 tumor stage, nevertheless, there was no significant correlation between the spontaneous immune humoral responses and the pathological stages or Gleason score (Table 3). Interestingly, 5 of 9 (55.5%) mRNA NY-ESO-1 positive tumors develop a spontaneous humoral response against NY-ESO-1. Whereas, of the twelve antibody positive patients, seven (58.3%) do not express NY-ESO-1 mRNA in the tumor (Table 4).

Table 4 : Relationship between NY-ESO-1 mRNA expression and anti-NY-ESO-1 antibodies in sera of prostate cancer patients.

NY-ESO-1 mRNA expression	NY-ESO-1 antibody response	Number of cases
Positif	Negatif	4 (36%)
Positif	Positif	5 (42%)
Negatif	Negatif	7 (64%)
Negatif	Positif	7 (58%)

DISCUSSION

The recognition of tumor cells by the immune system is reflected by spontaneous cellular and humoral immune responses. The immune system can target and destroy tumor cells recognizing specific tumor antigens [15]. Based on autologous T cell and antibody responses in cancer patients, a growing number of tumor antigens have been identified. To date, only few of these antigens, e.g. NY-ESO-1, have been reported to frequently elicit spontaneous cellular and humoral immune responses. This antigen remains one of the most immunogenic cancer testis antigen [5, 6, 16, 17].

In the present study, we investigated NY-ESO-1 expression and immunogenicity in PC. Initially, we performed an RT-PCR and revealed that NY-ESO-1 is expressed at relatively high frequency in PC patients (40%), while no NY-ESO-1 mRNA was observed in BPH. In comparison with other studies, which were carried out in PC, we observed in some extent concordant results. Indeed, Nakada et *al*. demonstrated the expression of mRNA NY-ESO-1 in 38% (20/53) PC patients. However, other studies showed striking frequencies of NY-ESO-1 protein expression. While a rare expression of NY-ESO-1 protein with 8% (9/114) rate in PC patients was found by Fossa et *al* , Hudolin's study showed an expression by 85% (79/92) of cases [18]. This discrepancy could be related to the fact that CTA are often focally expressed [19, 20]. The use of bioptic materials might result in an underestimation of NY-ESO-1 expression in PC patients.

There is a tendency for NY-ESO-1 mRNA expression in primary tumors to be associated with lymph node or systemic metastasis [5, 8, 21]. In our study, no significant correlation between NY-ESO-1 mRNA expression with clinical stage was found (T1-T2: 28,5%, T3-T4: 44%). These results are not in line with Fossa's findings which demonstrated that NY-ESO-1 expression protein is higher in advanced PC (7/48) then in localized PC (2/66) [22]. However, we did not find a significant correlation between NY-ESO-1 mRNA expression with the Gleason score (<7: 44%, ≥7: 35%), which is consistent with Nakada's findings [23]. The Immunogenecity of NY-ESO-1 has been demonstrated in several cancers. Noteworthy, NY-ESO-1 is the most immunogenic antigen defined to date. The availability of NY-ESO-1 full-length recombinant protein allowed us to investigate the NY-ESO-1 status in 23 PC and 23 BPH patients. Our results showed the presence of specific IgG antibodies anti-NY-ESO-1 in 52% PC patients and in 55,5% of NY-ESO-1 mRNA positive patients. Nakada *et al* and fossa *et al* have reported a frequency of 4,6% and 10% respectively [22, 23]. The reason for this difference is unclear but could be due to genetic variability between ethnic groups.

In several cancers as, melanoma, hepatocarcinoma, breast and lung cancer NY-ESO-1 antibody response as well is highest in advanced stages [13]. This tendency is not observed in PC. Indeed, in accordance with Fossa *et al* [22], we showed that NY-ESO-1 antibody responses is not significantly higher in locally advanced and metastatic stages (T3-T4: 50%) than in localized stages PC (T1- T2: 71,4%). A correlation study between NY-ESO-1 antibody and clinical features and disease outcome in large series of PC patients might help to clarify the role of the immune response in this cancer and provide useful information on the prognosis.

We had initially expected that NY-ESO-1 antibodies would be expressed by NY-ESO-1 mRNA positive tumor patients. However, of the twelve antibody positive patients, seven had tumors that were negative for NY-ESO-1 mRNA expression. This result could be explained for one patient by the expression of LAGE-A1 in the tumor and the high homology existing between this antigen and NY-ESO-1. For the rest, this result may be explained by the heterogeneity of expression of NY-ESO-1 by the tumor cells or by the presence of an unknown NY-ESO-1 expressing metastasis. It can also be speculated that the tumor was originally NY-ESO-1 positive and the elicited anti-NY-ESO-1 response leads to the clearance of the NY-ESO-1 positive tumors, leaving NY-ESO-1 negative tumor cells unaffected.

In conclusion, in the present study even realized with a small sample, we clearly demonstrated an NY-ESO-1 mRNA expression and NY-ESO-1 antibody response in PC patients. Although the enhancement of NY-ESO-1 protein expression and the activation of immune response of the patient with PC are necessary, NY-ESO-1 has the potential to be a good target molecule for immunotherapy against hormone-refractory PC, for which the effectiveness of current therapeutic modalities is limited. This preliminary study needs enlargement of the sample and investigation of the immune response against NY-ESO-1 and its follow at different stages of the PC disease in correlation with mRNA and protein expression in the tumors. This could give data allowing the use of NY-ESO-1 as a prognosis marker and a target for immunotherapy.

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