



ISSN: 2277- 7695

TPI 2016; 5(4): 20-26

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www.thepharmajournal.com

Received: 17-02-2016

Accepted: 19-03-2016

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Antibody detection against HPV16 E7 & GP96 fragments as biomarkers in cervical cancer patients

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Abstract

Background & objectives: Cervical cancer is the second most frequent cancer among female's worldwide, especially human papilloma viruses (HPV) types 16 and 18. In viral systems the identification of serological markers would facilitate the diagnosis of HPV infections and virus-related disease. The aim of the present investigation was to determine and search for serologic markers in cervical cancer patients associated with HPV.

Methods: A total of 58 Iranian women with invasive cervical carcinoma including adenocarcinoma and squamous cell carcinoma (SCC) were included. Serum antibody response to HPV infections in patients was detected by Western blot and ELISA techniques based on recombinant HPV16E7 and the N-terminal and C-terminal fragments of gp96 (NT-gp96 and CT-gp96) proteins. These recombinant proteins were expressed in *Escherichia coli* as a His-tag protein and purified using affinity chromatography.

Results: The ELISA results indicated that patients with high antibody response to HPV16E7 had significant seroreactivity to CT-gp96 fragment. In Western blot analysis, a strong association between anti-E7, anti-NT-gp96 and anti-CT-gp96 reactivity and cervical cancer was obtained using purified recombinant proteins. In adenocarcinoma cases, no significant difference was observed in seroreactivities between normal and patients.

Interpretation & conclusions: The evaluation of cervical cancer patients' seroreactivities against three recombinant proteins (rE7, rNT-gp96 and rCT-gp96) showed significantly higher levels of these markers in SCC only, but not in adenocarcinoma and control groups. Also, the usage of both techniques (ELISA and Western blotting) can provide more reliable tools for diagnosis of cervical cancer.

Keywords: Cervical cancer - E7 - GP96 - HPV - serological marker

Introduction

Cervical cancer is a major cause of death in woman of reproductive age in different parts of the developing world¹. High-risk human papillomaviruses (HPVs) infections mainly HPV types 16 and 18 are a good predictor of subsequent high grade lesions in young woman². The oncogenetic mechanism of HPV can be explained by the regulation and function of the two viral oncogenes E6 and E7. E7 is the major.

Transforming protein produced in cervical cancers and therefore represents potential tumour specific antigen that could be the target of immunotherapy for cervical cancer^[1, 3, 6]. Antibodies against the E6 and E7 proteins of HPV types 16 and 18 have been found to be strongly associated with cervical cancer but the value of E6 and E7 specific serology for the diagnosis of this disease is still questionable^[2, 7-12]. Heat shock proteins (HSPs) perform essential biological functions under both physiological and stressful conditions^[13, 19]. GP96 is the most abundant member of HSP90 in the endoplasmic reticulum (ER), contains an N-terminal signal sequence (70 amino acid), characteristic of the ER-targeted proteins and a carboxyl terminal KDEL sequence (Lys-Asp-Glu-Leu), which is a retention/retrieval signal from the golgi to the ER. Immunization with HSP complexes isolated from tumour or virus infected cells has been shown to induce potent antitumour or antiviral immunity.

The gp96 fragments (NT-gp96 and CT-gp96) as mini-chaperones are better choice for immunization. Extracellular release of HSPs upon necrotic cell death and their modulated access at the surface of some cells can be considered as a putative danger signal. Also, gp96 promoter is activated in a variety of spontaneous or induced tumours, suggesting that tumour microenvironment is a potent physiological inducer of gp96^[20-23].

Since HPV cannot be propagated in tissue culture to purify oncoproteins, therefore, it will be based on production of recombinant proteins in viral or bacterial expression systems. In this

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study, the recombinant proteins of E7, NT-gp96 and CT-gp96 (rE7, rNT-gp96 and rCT-gp96) were expressed in *Escherichia coli* and seroreactivities of patients with invasive cervical cancer against these recombinant proteins were examined as diagnostic markers.

Material & Methods

Recombinant E7, NT-gp96 and CT-gp96 proteins production

The E7 gene was digested from the pcDNAE7 encoding E7 protein from human papillomavirus type16 (kindly provided by Prof. T.C.Wu, John Hopkins Medical Institutions, USA) using *Bam*HI and *Hind*III and the resulting DNA fragment was gel purified according to manufacturer details (QIAquick gel extraction kit protocol, QIAGEN, USA). The digested product was cloned into the expression vector pQE30 (QIAGEN) which encodes the N-terminal 6XHis-tag. The resulting plasmid was referred to as pQE-E7. The *E.coli* M15 strain (QIAGEN, USA) was transformed with pQE-E7 and grown at 37 °C in Luria Bertani (LB) broth supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin. For the production of recombinant E7 (rE7), the cultures were grown to an optical density of 0.6-0.8 at 600 nm (OD₆₀₀= 0.6-0.8), and protein expression was induced with 1 mM isopropyl-beta-Dthiogalactopyranoside (IPTG) at 37 °C. The cell pellets were collected and stored at -20 °C until used for protein purification.

The N-terminal and C-terminal fragments of gp96 genes were obtained from the pBluescript II vector containing the coding sequence of gp96 protein from *Xenopus laevis* named Xgp10 (a kind gift from Dr Jacques Robert, University of Rochester Medical Center, USA). The DNA encoding the N-terminal and C-terminal fragments of gp96 consisted of 1014 (from 64-1077 nucleotide) and 907 (from 1645-2552 nucleotide) base pairs, respectively, were cloned into the *Bam*HI and *Kpn*I sites of expression vector pQE30. For obtaining different truncated forms of NT-gp96 and CT-gp96, four pairs of primers (MWG Biotech GmbH, Germany) were designed (the *Bam*HI and *Sac*I & *Kpn*I restriction sites have been shown in bold). The forward and reverse primers for amplifying the NT-gp96 (NT1 & NT2) and CT-gp96 (CT1 & CT2) were designed as follows:

NT1: 5'-CGGGGATCCGAAGATGACGTTGAA-3'
 NT2:5'-ATGAGCTCGGTACCTTTGTAGAAGGCTTTGTA - 3'.
 CT1: 5'-GGGGATCCGAGTCTCTCCATTGTGAA- 3'
 CT2: 5'-ATGAGCTCGGTACCCGGCCGCGAAAGGTTTTTA- 3'

PCRs were performed by PCR thermal cycler [24], (Techne, USA) under standard conditions (94 °C for 1 min; 62 °C for 2 min and 72 °C for 1.5 min for 30 cycles) and the products were separated on a 0.8 per cent agarose gel. The bands corresponding to the expected PCR products size were gel purified (QIAquick gel extraction kit protocol, QIAGEN), digested with *Bam*HI and *Kpn*I and ligated into a similarly digested expression plasmid pQE30. The ligation mixture was used to transform *E.coli* M15 strain. The resulting clones were confirmed by sequencing (the dideoxy chain termination method on an automated sequencer). The proteins (NT-gp96 and CT-gp96) were induced individually using 1 mM IPTG.

Protein samples were analyzed by SDS-PAGE in a gel containing 15 per cent (W/V) polyacrylamide for HPV16E7 and 12.5 per cent (W/V) polyacrylamide for NT-gp96 and CT-gp96 (SDS gel apparatus; BioRad, USA), followed by staining with coomassie brilliant blue. For Western blot analysis 25, the proteins resolved on the gel were transferred onto protran

nitrocellulose transfer membrane (Schleicher and Schuell Bioscience, Dassel, Germany). The membrane was pre-equilibrated with Tris-buffered saline Tween-20 (TBST) solution [10 mM Tris-HCl (pH 7.4), 150mM NaCl, and 0.1 per cent Tween 20] containing 2.5 per cent bovin serum albumin (BSA) for overnight and then reacted with anti-His antibody (QIAGEN, USA) for 2 h at room temperature. After three washes with TBS, the membrane was incubated with anti-mouse IgG-HRP (1:2000, Sigma, USA) for 1.5 h at room temperature. The immunoreactive protein bands were visualized using peroxidase substrate named 3, 3'-diaminobenzidine (DAB, Sigma, USA). For the recombinant E7 protein, mouse anti-HPV16E7 (monoclonal antibody, USBiological) instead of anti-His antibody was used and the same protocol was followed.

Purification and assay of the recombinant proteins

Proteins were purified by affinity chromatography on Ni-NTA resin (nickel-nitrilotriacetic acid) using 6×His-tag. Initially, E7 protein solubility was determined. Based on SDS-PAGE analysis, the expression of E7 protein was found to be almost equally disturbed between soluble and insoluble fractions. Purification of recombinant E7 protein was done under native and denaturing conditions. Fast protein liquid chromatography (FPLC) purification of 6xHis-tagged E7 using Ni-NTA column (Amersham Pharmacia Biotech, QIAexpress Protein Purification System, Hand book, QIAGEN, USA) was performed under denaturing condition. Also, the soluble fraction of rE7 was purified using a Ni-NTA resin under native conditions. The recombinant NT-gp96 and CTgp96 proteins were mostly soluble and were purified by Ni-NTA chromatography under native condition according to the manufacturer's protocol (QIAGEN). Briefly, the recombinant proteins can be purified by Ni-NTA chromatography based on the interaction between a transition Ni²⁺ ion immobilized on a matrix and the histidine side chains. Following matrix washing, 6xHistidine-tag fusion proteins were eluted by adding free imidazole or EDTA or by reducing the pH. The purified protein fractions were concentrated by ultrafiltration (Amicon, USA), and dialyzed against PBS (dialysis membrane, MWCO: 3.500 kDa, Spectrum). The protein concentrations were measured by the BCA procedure (Pierce, Rockford, USA) using bovine serum albumin (BSA) as a standard and stored at -70°C.

Human serum samples

Serum samples were collected from 58 Iranian women from March 2007 for six months in total (in clinical stage I without any previous treatment) with clinically diagnosed and histopathologically confirmed as invasive cervical cancer with no previous treatment (Imam and Valiasr hospitals, Tehran, Iran) and age matched control subjects stored at -20°C. Cervical scrapes were used to prepare a Pap smear within 6 months (Cancer Institute, Imam Hospital, Tehran, Iran). In this study, the mean age of Iranian patients was 47.8 yr (range, 22 to 78 yr), and the mean age of Iranian controls was 44.2 yr (range, 25 to 68 yr). The pathologic studies showed that ten patients were suffering from adenocarcinoma and the remaining (48 cases) had squamous cell carcinoma (SCC). Controls were recruited from commercial standard sera (USA origin: Innovative Research, INC) and 10 Iranian women with no previous infection with HPVs. All participants gave written informed consent, and the Pasteur Institute of Iran Ethics Committees cleared and approved the study protocol. Antibody

detection against specific and conserved viral protein (HPV16E7) and different fragments of Gp96 was done in limited samples from cases and controls. E7 protein has been proposed as HPV marker for several years [2, 7-12], but similar antibody response has been observed in some normal subjects (without HPV infections) [2, 7-12].

ELISA and Western blotting with human sera: Each well of 96-well microtiter (NUNC, Germany) was coated overnight at 4 °C with 100 µl of the antigens; rE7 (5 µg/ml), rNT gp96 (2.5 µg/ml) and rCT-gp96 (10 µg/ml), all in phosphate buffer saline (PBS), pH 7.2. Then, the plates were blocked with 1 per cent BSA in PBS for 2 h at 37 °C and incubated with 100 µl of human sera diluted 1:50 in blocking buffer [PBS-1 per cent BSA-0.05 per cent (v/v) Tween 20] for 2 h at 37 °C. Bound human antibodies were detected by goat anti-human (IgG, IgA and IgM, Sigma) polyclonal antibodies conjugated to horseradish peroxidase (HRP) (diluted 1:1000 in blocking buffer and incubation for 2h at 37 °C) by using O-Phenylenediamine (OPD, Sigma) as the substrate. All washing steps were done with PBS containing 0.05 per cent Tween 20. After 30 min, the enzyme reaction was stopped by adding 50 µl of 1M sulphuric acid and the absorbance at 492 nm was determined.

For Western blot analysis, the same procedure was taken as described for anti- His antibody with minor differences. The human serum samples (invasive cervical cancer patients and controls) with 1:50 dilutions were used. After three washes with TBST, the membrane was incubated with anti-human poly antibody-HRP (sigma) for 1.5 h at room temperature. The immunoreactive protein bands were visualized. The detection was performed using Western blotting detection reagent (Sigma, USA). Quantity-One Software (Bio-RAD, USA) was used for relative quantity comparison of recombinant proteins between patient and control serum samples.

Statistical analyses: The differences in the level of antibody production were determined by one-way ANOVA. $P < 0.05$, was considered significant.

Results

Expression and purification of recombinant HPV16E7, NT-gp96 and CT-gp96 proteins: The recombinant E7 protein consisting of 98 amino acid residues of the fulllength E7 gene was expressed and purified in *E. coli*. The rE7 migrated as a 23 kDa protein in SDS-PAGE (Fig.1 A). A molecular mass of the 23 kDa protein was larger in size than predicted (11kDa of E7 protein plus 4 kDa protein of His-tagged regions in the pQE vector system) [6, 26, 27]. In Western blot experiment, the existence of two additional bands for rE7 (about 28 and 40 kDa) was observed which may due to alternative conformational forms of E7 protein (Fig.1 A). The protein of interest was both in the insoluble and the soluble fraction;

therefore, purification was performed under denaturing and native conditions using affinity chromatography. Similar steps as HPV16E7 were taken for the Nterminal and C-terminal fragments of gp96. The rNT rNTgp96 and rCT-gp96 migrated as about 43 and 34kDa proteins in SDS-PAGE, respectively (Fig. 1 B & C). Western blot analysis was done for both NT-gp96 and CT-gp96. The 6XHis-tag in rCT-gp96 is partially hidden. Therefore, longer binding times (overnight at 4 °C) were used for anti-His antibody (Fig. 1 B & C). Purification of rNT-gp96 and rCT-gp96 were done under native condition.

Detection of HPV16E7-specific antibodies by ELISA and western blotting: The frequency of antibodies to HPV16E7, NT-gp96 and CT-gp96 was determined in 58 serum samples from Iranian patients clinically diagnosed with invasive cervical cancer and commercial standard sera in addition to 10 normal women from Iran. The mean absorbance values for rE7, rNT-gp96 and rCT-gp96 were 1.012 ± 0.547 , 0.660 ± 0.198 and 0.630 ± 0.202 , respectively (fig. 2). To determine the serum reactivity for each recombinant protein, a cut-off value was calculated by considering the mean absorbance values of control sera plus two standard deviations (mean \pm 2 SD). Overall, 50 per cent of the serum samples from patients with cervical carcinomas reacted in both assays as anti-E7 and anti-CT-gp96 antibody positive and 32.7 per cent of the sera recognizing E7 and NTgp96 proteins. Also, 17.2 per cent of the serum samples were only positive to CT-gp96 protein. There were significant differences between patient and controls in respect to rE7 and rCT-gp96 ($P < 0.05$). The frequency of anti-E7, anti-NT-gp96 and anti-CT-gp96 antibody in patients suffering from adenocarcinoma (A) was less, not significant, than patients with squamous cell carcinoma (SCC) (Table). In Western blotting, the determination of relative quantity of rE7, rNT-gp96 and rCT-gp96 bands showed considerable difference between patient and control serum samples. The mean relative quantity values for rE7, rNT-gp96 and rCT-gp96 in SCC patients were 50.6 ± 0.761 , 31.3 ± 0.419 and 26.8 ± 0.738 , respectively. Relative quantity for the patients suffering from SCC was significantly higher than normal serum samples (as controls) and the patients suffering from adenocarcinoma ($P < 0.05$) (Fig. 3). No visible bands were observed in control serum samples at 23, 43 and 34 kDa corresponding to rE7, rNT-gp96 and rCTgp96 bands, respectively. The bacterially expressed recombinant proteins of rE7 and rCT-gp96 appeared as a predominant band at 23 and 34 kDa respectively, but an extra band was also seen in the purified protein fractions (Fig. 4 A & B). These additional bands have been observed in some patients. Also, two additional bands were detected in the purified NT-gp96 protein (Fig.4 B). These extra bands are likely related to alternative conformational forms of three recombinant proteins that were detected in some patients.

Table. Serum reactivity in patients and controls with each antigen using ELISA method

Human subjects/ antigens	SCC (N= 48)	A (N= 10)	Total (SCC + A)	Normal (N= 11)
rE7	1.030 \pm 0.586*	0.897 \pm 0.129*	1.012 \pm 0.547*	0.735 \pm 0.078
rNT-gp96	0.666 \pm 0.202	0.624 \pm 0.169	0.660 \pm 0.198	0.570 \pm 0.043
rCT-gp96	0.629 \pm 0.209*	0.637 \pm 0.159*	0.630 \pm 0.202*	0.448 \pm 0.048

SCC, Squamous cell carcinoma; A, adenocarcinoma
* $P < 0.05$ compared to normal. Values are mean \pm SD

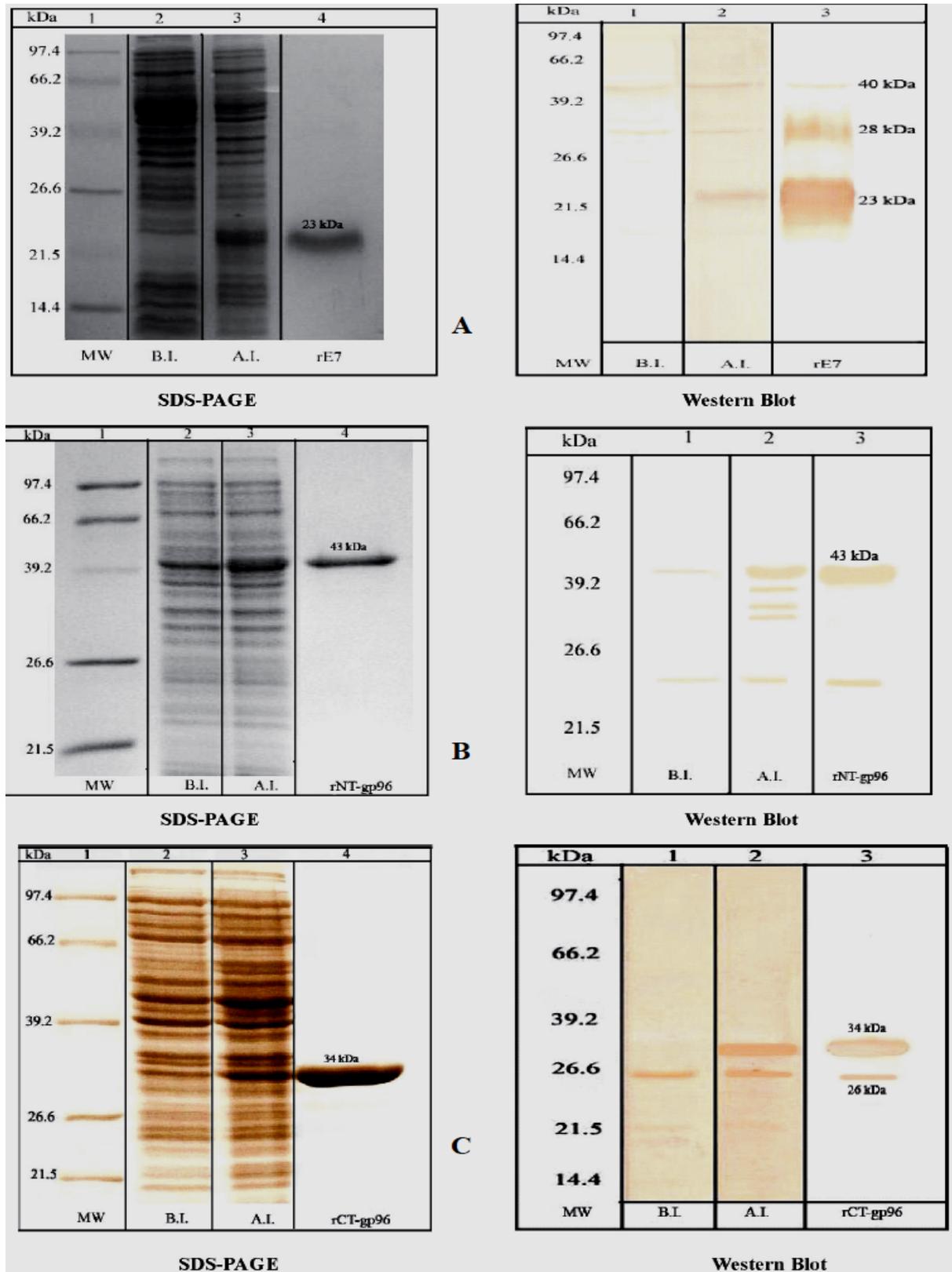


Fig 1: Expression and purification of the recombinant proteins.

The genes were cloned into the pQE-30 expression vector. Analysis of recombinant proteins was done by SDS-PAGE and Western blotting. The recombinant HPV16E7 (A), NT-gp96 (B) and CT-gp96 (C) proteins were run on a polyacrylamide gel electrophoresis (15% for E7, 12.5% for both NT-gp96 and CT-gp96) and stained by coomassie blue. The weight of the molecular mass markers (MW: Premixed protein molecular weight marker: 14.4- 97.4 kDa) is indicated on line 1 in each figure. Lysate of uninduced and isopropyl-beta-D-

thiogalactopyranoside (IPTG) (1 mM) induced culture of *E.coli* is shown as B.I. (Before Induction) and A.I. (After Induction) on line 2 and 3, respectively. Purified recombinant proteins by affinity chromatography using Ni-NTA resin are indicated on line 4. Also, Western blot analyses for each recombinant protein are indicated on the right of panel (A, B and C) as compared with SDS-PAGE. The existence of extra bands for rE7 and rCT-gp96 has been shown.

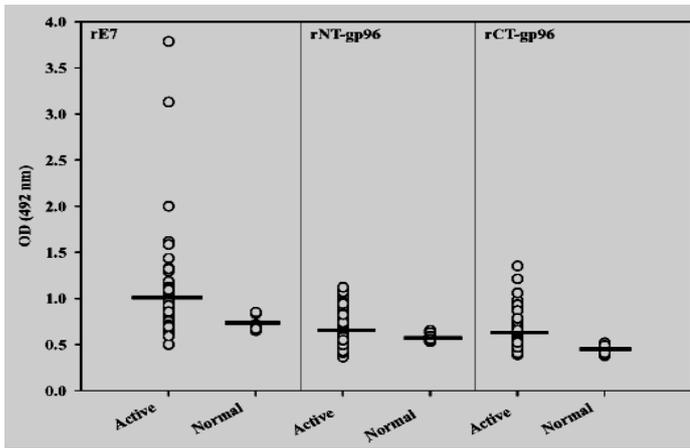
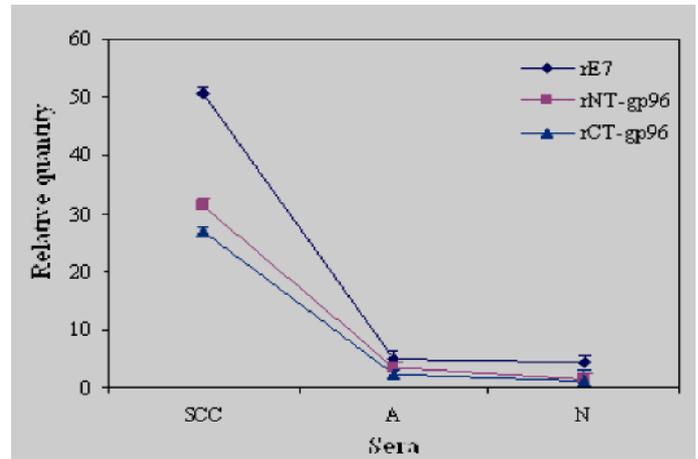


Fig 2: ELISA based on HPV16E7, NT-gp96 and CT-gp96 coating antigens. Detection of antibodies to these recombinant proteins in 58 serum samples from Iranian patients clinically diagnosed with invasive cervical cancer in comparison with normal subjects is shown. The coating antigens are indicated on each panel. The horizontal line represents the mean value of optical density in respect to each antigen. Active samples include squamous cell carcinoma (SCC) and adenocarcinoma patients sera.



* $P < 0.05$ compared to adenocarcinoma and control group.

Fig 3: Relative quantity of seroreactivity in patients suffering from squamous cell carcinoma (SCC) and adenocarcinoma (A) as compared with control group (N) using Western blotting against different recombinant proteins. Relative quantity for the patients suffering from SCC was significantly higher than normal and the patients suffering from Adenocarcinoma ($P < 0.05$).

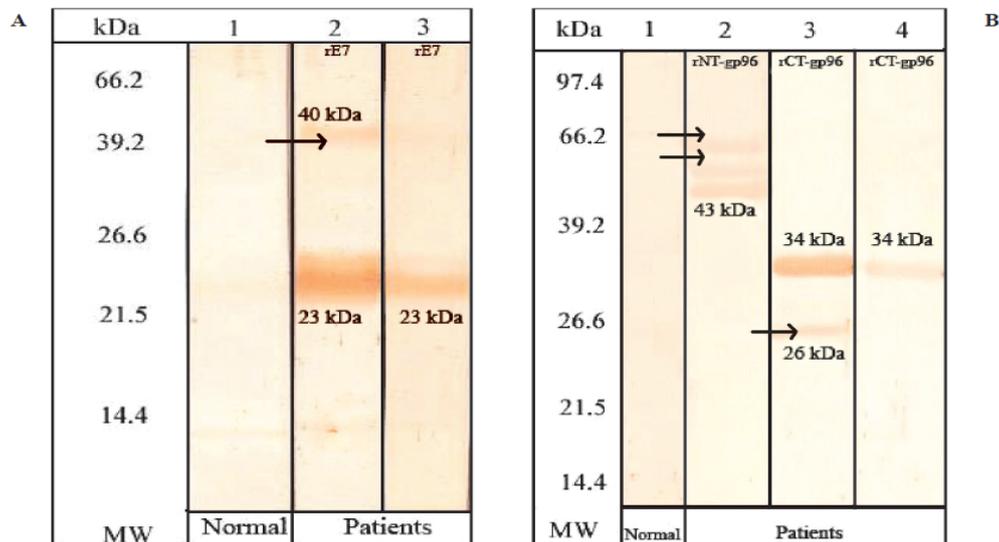


Fig 4: Western blot analysis of different human serum samples.

Molecular weight marker is indicated on the left side as kDa. Samples were tested at 1:50 dilution. Patient sera that are positive for antibodies to rE7 (A), rNT-gp96 (B) and rCT-gp96 (B) are shown in lines 2 and 3 (A), line 2 (B) and lines 3 and 4 (B), respectively. The existence of the additional bands for rE7, rNT-gp96 and rCT-gp96 has been marked by arrows. Antibody responses to rNT-gp96 were similar in patients. The anti-HPV16E7, anti-NT-gp96 and anti-CT-gp96 responses were not observed in the control sera as shown in line 1 (A and B).

Discussion

In this study, the presence of human antibodies to HPV16E7 and two fragments of heat shock protein (gp96) were demonstrated by both Western blotting and ELISA. Recombinant E7, NT-gp96 and CT-gp96 proteins expressed in *E. coli* were used as antigens in Western blot and ELISA to screen 58 serum samples obtained from patients with cervical cancer as well as controls. In order to achieve these goals, the HPV16 E7 gene was cloned into the bacterial expression vector (pQE-30). Electrophoresis analysis of IPTG-induced cell lysates showed the presence of a prominent protein band that was not

detectable in non-induced cell lysates. In the presence of sodium dodecyl sulphate and 2- mercaptoethanol, the E7 protein migrated as a 23kDa protein during poly acrylamide gel electrophoresis. However, the theoretical molecular mass of this protein is approximately 11kDa. These results indicate that the substantial net negative charge of the wild type E7 protein is responsible for its anomalous electrophoretic behaviour. This electrophoretic behaviour of the HPV16E7 protein is associated with the amino terminal half of the protein as described previously [26, 27]. Based on SDS-PAGE analysis, the expression of E7 protein was found to be almost equally disturbed between soluble and insoluble fractions. HPV infects squamous epithelium with expression of various gene products intimately linked to epithelial cell differentiation. Hence, there are basically three classes of detectable markers directly derived from HPVs: molecular markers based on detection of nucleic acid sequences, serological markers based on detection of antibodies against viral proteins, and cellular markers based on detection of proteins expressed intracellularly, upon either infection or carcinogenesis [28]. There is an increasing demand to develop standard tools to assess the quality of HPV detection systems for clinical purposes. In this study, the gp96 fragments (NT-gp96 and CT-gp96) were used as serological markers, in addition to HPV16E7. Heat

shock proteins (HSPs) including gp96 are highly conserved intracellular molecular chaperones. Cell surface expression of heat shock protein gp96 enhances cross-presentation of cellular antigens and the generation of tumourspecific T cell memory [13-15, 29-31]. The changes in HSP levels have been shown in some diseases including parasite, virus and tumour model systems. But the quantity of immune response to gp96 has been rarely studied. Also, there are no reports related to cervical cancer. In this study, DNA encoding the N-terminal and C-terminal fragments of gp96 was cloned into the expression vector pQE30. These fragments were soluble and purified through native method by Ni-NTA system. SDS-PAGE and Western blot analyses were used to confirm the identity of them. The rNTgp96 and rCT-gp96 migrate as 43 and 34 kDa proteins, respectively.

Different studies have shown that in adenocarcinoma, HPV prevalence was significantly lower than that in SCC, and the HPV18 was the predominant type in every region followed by HPV16 and HPV4532. Some reports from Sweden, United States and Australia have indicated that the incidence of invasive cervical adenocarcinoma, which used to account for 10-15 per cent of all cervical cancers, has been steadily increasing in young women, even as the overall incidence of cervical cancer has declined [33]. The cause of the increase is unclear, but the reports have shown a poorer prognosis for patients with cervical adenocarcinoma than for those with squamous cell carcinoma. At diagnosis, adenocarcinomas tend to be larger and exhibit a propensity for early lymphatic and haematogenous metastasis [33]. As adenocarcinomas start in the cervical canal, these can be more difficult to detect with cervical screening. Effective screening programmes have contributed to a decrease in the incidence of cervical squamous cell carcinomas but have had a limited sensitivity in the detection of adenocarcinoma precursor lesions³⁴. Our findings indicate that patients with an anti-HPV16E7 response had a high reactivity to rNTgp96 or rCT-gp96, but there was no seroreactivity in control sera. Also, in adenocarcinoma cases, there was no significant difference in seroreactivities between normal and patients. Our data obtained clearly in Western blot experiments suggest that the significant higher levels of three recombinant proteins can be used as markers in SCC only, but not in adenocarcinoma and control groups. Therefore, heat shock proteins besides viral oncogenic proteins could be used to screen the patients suffering from SCC.

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