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Research Article

Immunotherapy of Prostate Cancer may Change mRNA Level of Virulence Factor Genes in *E.Faecalis* of Microflora

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Abstract | **Background:** FDA has approved the immunotherapy for prostate cancer in 2010. Immunotherapy is the treatment of disease by inducing, enhancing, or suppressing an immune response and is well known as specific and noninvasive method for cancer treatment; but side effects of this method are not clarified. Enterococci are Gram positive coccies, which are intestinal commensals and microflora of humans and other mammalians. Most enterococcal infections in human such as gastroenteritis, intestinal infections, prostatitis and endocarditic are causing by *Enterococcus faecalis*. Present study aimed to evaluate the side effects of immunotherapy on *E. faecalis* of microflora. **Methods:** mRNA level of 10 virulent genes (*gelE*, *esp*, *asa1*, *aggA*, *cylA*, *cylB*, *cylM*, *Eep*, *efaA* and *enlA*) which are involved in pathogenesis of *E. faecalis*, were examined in stool samples of two groups of men by quantitative real time PCR. Group A includes 359 prostate cancer patients and group B contains 360 normal family members of patients, which were lived with them at least for recent 12 months. Gene expression assessments in patient's group were operated before start and after finishing a six weeks' period of cancer vaccines immunotherapy. **Results:** Results were showed significant (P<0.05) over expression of 8 genes *(gelE*(P=0.001). *asa1*(P=0.001), esp (P=0.002), *aggA*(P=0.001), *efaA*(P=0.002), *enlA*(P=0.001), *cylA*(P=0.003) *and cylB*(P=0.003) in patients after treatment compared to before treatment. Also significant over expression of these 8 genes has been detected in patients after treatment in compare with normal related subjects. No significant alterations were observed in expression of virulence genes between normal subjects and patients before treatment. **Conclusions:** it seems that immunotherapy may carry side effects such as increasing the pathogenicity risk of microflora in treated patients. These side effects could cause further infections after ending the immunotherapy of cancer. Based on these results, antibiotic treatments after immunotherapy for prevention of potential infections could be recommended.

Keywords | Prostate cancer, Immunotherapy, Side effects, *Enterococcus Faecalis*

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INTRODUCTION

Immunotherapy is a new treatment method of diseases by inducing, enhancing, or suppressing of natural immune response. Activation immunotherapy which is designed to elicit or amplify an immune response is widely used for cancers' treatment , and suppression immunotherapy that reduce or suppress the immune system mostly use for treatment of allergies (Vatsan et al., 2013). Immunotherapy for cancer primarily was introduced by Rosenberg in National Institutes of Health in the United States. Prostate cancer was one of the first cancers that achieved

approval by the FDA for immunotherapy, in 2010. Prostate cancer is the second most common cancer in men, and the eighth cause of cancer-related death around the world. There are almost 1,100,000 new prostate cancer cases and 300,000 mortalities worldwide every year that is four percent of all cancer deaths. It is estimated that one in every six men will be diagnosed with the disease during his lifetime (Gerritsen, 2012). In United States of America; more than 90% of prostate cancers are found in local or regional stages (Gerritsen, 2012).

Several immunotherapeutic strategies are being investigated in clinical trials of prostate cancer patients including therapeutic vaccines, oncolytic virus therapies, checkpoint inhibitors, adoptive cell therapies and adjuvant immunotherapies (Hoos, 2012). Although immunotherapy is a noninvasive and useful treatment method for cancer therapy, side effects of this method still is not completely clarified (Tayebe Talebzade, 2017). One of the possible side effects of every cancer treatment method in human is deregulating of microbial flora functions and orders. Side effects of common cancer treatments such as radiotherapy and chemotherapy on microbial flora of patient's body including decreases in the number and diversity of microbiota and susceptibility of under treatment's patients to infections such as gastrointestinal mucositis had reported in patients and rat models (Fijlstra et al., 2015, Tayebe Talebzade, 2017, Nicolatou-Galitis et al., 2006); but to best of our knowledge there is no study about immunotherapy microbial side effects. Post-treatment infections in prostate cancer patients are a major problem and most reported infections in these patients are prostatitis commonly induced by *E. Coli* and *Enterococcus faecalis* (Seo and Lee, 2013, Liss et al., 2011). This could be because of treatment's effects on pathogenicity of these microorganisms.

Enterococci are Gram-positive coccies and intestinal commensals of humans and other animals, in addition to be an isolate from environmental sources. Most enterococcal infections in human such as gastroenteritis, intestinal infections and endocarditic have been caused by *E.faecalis* (Comerlato et al., 2013). Although virulence factors of Enterococcus as opportunistic pathogens still are not completely detected, but several enzymatic or regulatory proteins with known functions were identified as pathogenicity factors in clinical and commensal *Enterococcus* isolates. Presence and increase in expression levels of virulence gene are significantly associated with infection, biofilm construction and antibiotic resistance in *Enterococcus* isolates (Semedo et al., 2003). The mRNA level of virulence genes could alter by several environmental and biological changes such as sub-lethal environmental stress, antibiotic treatment and number of host related events like surgeries or immune suppressor medications (Lenz et al., 2010). Expressions level evaluation of virulence genes is a useful tool for assessment and prediction of virulence state of microorganisms.

Present study aimed to investigate the side effects of immunotherapy in microbial flora pathogenicity by using quantitative Real Time PCR for study the expression level of most common virulence genes in *Enterococcus faecalis* of microbial flora in stool samples of prostate cancer subjects which are treated by immunotherapy.

MATERIAL AND METHODS

Subject Selections and Stool Sampling

Patient group was including 359 selected from early diagnosed prostate cancer patients which starting immunotherapy as first treatment method. All patients received six infusions of dendritic cells (DC) pulsed with PSM-P1 and -P2 at 6-week intervals based on Murphy et al study (Murphy et al., 1999). As microbial flora is strongly related to life style of individuals, 360 normal subjects selected from family members of patients, which were also lived with them at least for recent 12 months. Patient group and group of normal subjects matched in age, BMI and socioeconomic situation. None of the subjects had current or history of sever medical condition including any infection or allergy. All subjects were given an explanation on the purpose of the study and next, written informed consent has been provided.

From 359 treated patients, 116 patients were showed symptoms of prostatitis infection between 2 and 5 weeks after immunotherapy period. All 116 patients treated by ciprofloxacin (Cipro) for 4 weeks. Symptoms of infection in 23 patients did not reduce after 4 weeks' ciprofloxacin treatments. These 23 patients then treated with Vancomycin (Van) for 4 weeks, which decreased infection symptoms.

Stool sampling was operated before starting treatment and day after finishing treatment period. Fecal specimens were collected in plastic containers from subjects and were placed in the refrigerator in -70 centigrade, transported to the laboratory, and put on RNA extraction process immediately.

Gene Expression Study by Quantitative Real Time Pcr

Total RNA was extracted directly from fecal specimens according to standard protocol of stool sampling by QIAamp® Viral RNA Mini Kit (catalog number: 52906, QIAGEN®, USA). Quality of extracted RNA examined by 1% Agarose gel electrophoresis. The cDNA was synthesized using a Transcription first strand cDNA synthesis kit (RevertAid Premium First Strand cDNA Synthesis

Kit #K1652, Thermo Scientific, Latvia) according to manufacturer's protocol. Specific primers for all genes, including 10 genes of interest and 16srRNA as housekeeping gene designed by "oligo7" software and primer sequences blasted on NCBI website for checking of specific alignments to *E.faecalis* sequences. Quantitative RT-PCR in triplicate method performed by using CFX96 Touch Real-Time PCR Detection System (BIO-RAD, California, United States) and SYBR green kit (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) #K0221, thermo scientific, Latvia) according to manufacturer's protocol. The software plotted a standard curve of the cycle at threshold vs. extracted RNA quantity. Samples were measured in one plate for one target gene and their Ct values were in the linear range of the standard curve. Each outliers or failures samples were repeated for each gene. pffafle formula used for calculation of ratio.

Statistical Analysis

Descriptive data are expressed as mean ± SD (range) and level of statistical significance was set at $P < 0.05$. At the beginning normal distribution for continuous variables was assessed via the Kolmogorov-Smirnov test. Next, gene expression changes between before and after treatment groups were examined by paired t test and gene expression changes between patients and normal groups were analyzed by independent sample t test. Correlation of demographic and clinical data with gene expressions examined with Pearson correlation test. Bonferroni correction test was used for comparisons of multiple groups. SPSS (version 22) was used for statistical assessments.

Results and Discussion

Demographic, clinical and pathological data of patients are presented at Table 1. Age of average for patients group was 67±7.9 and for normal group was 67±5.4. Most of the patients were in stage B and took 6 in Gleason scale. No chronic patients were included.

Table 1: Demographic and clinical data for patients (PSA: Prostate-Specific Antigen test results, ABCD stage: stages of prostate cancer)

Age	PSA	Gleason scale	ABCD stage	prostate infection
67± 7.9	19.6± 8.5	288 patients: 6 GS 45patients: 7 GS 26 patients : 8 GS	316 patients: stage B 43 patients: stage C	no infection: 243 prostatitis treated by ciprofloxacin: 93 prostatitis resistant to ciprofloxacin: 23

Gene Expression Results

No significant difference was found in expression of any of 10 genes between patient's group samples and samples of their related normal subjects. significant over expression of *gelE*(P=0.001), *asa1*(P=0.001), esp (P=0.002), *aggA*(P=0.001), *efaA*(P=0.002), *enlA*(P=0.001), *cylA*(P=0.003) *and cylB*(P=0.003) genes has been detected in "after treatments" group in compare with "before treatments" group. Gene expressions of these 8 genes were significantly increased in after treatment group in compare with normal subjects. Summary of gene expression data are presented in Table 2, Table 3 and Table 4. No significant correlation were found between gene expression results and clinical or demographic data of patients and severity scales of prostate cancer including Gleason scale, Prostate-Specific Antigen (PSA) Test results and stage (ABCD) of disease in starting time of treatment.

Table 2: Gene expression ratio and *P-values* of *E.faecalis* microflora genes in patients before immunotherapy treatment vs. related normal subjects

Gene	P-values	Mean ratio
gelE	0.32	0.94±0.04
asa1	0.31	0.98±0.02
Esp	0.41	1.07±0.06
aggA	0.29	1.02±0.02
cylA	0.42	0.93±0.08
cylB	0.18	0.98±0.09
cylM	0.35	0.96±0.05
Eep	0.41	1.09±0.14
efaA	0.37	0.97±0.07
enlA	0.48	1.05±0.012

Table 3: Gene expression ratio and *P-values* of*E.faecalis* microflora genes in after immunotherapy treatments vs. before immunotherapy treatments

Gene	P-values	Mean ratio
gelE	0.001	1.94±0.22
asa1	0.001	1.87±0.16
esp	0.002	1.78±0.18
aggA	0.001	1.87±0.06
cylA	0.003	1.83±0.15
cylB	0.003	1.79±0.07
cylM	0.14	1.15±0.23
Eep	0. 06	1.22±0.19
efaA	0.002	1.72±0.18
enlA	0.001	1.96±0.21

Several studies approved effectiveness and specific effect of immunotherapy, but still there is lack of studies about side effects of this method (Hoos, 2012, Tayebe Talebzade, 2017). Increasing number of reports about prostate inflammation and prostatitis in Prostate cancer patients

who finished a period of immunotherapy was the origin motivation of present study. As patients were living in isolated situation and possibility of external infection was at the minimum, researchers hypothesized that microflora in patients may became virulent. Enterococci are commensal lactic acid bacteria that are associated with human digestive tract and also are presented in prostate (Virji et al., 2015). Previous studies were showed virulence genes activities are major factors, which convert Enterococci from a harmless bacterium to virulent microorganism (Repizo et al., 2014, Radhouani et al., 2014, Bhardwaj et al., 2017). Results of present study have been showed influence of immunotherapy on virulence factors of microbial flora. No significant difference was detected in patients in compare with related normal individuals which means cancer would not change gene expression pattern of virulence genes in microflora. Over expression of all 10 virulence genes in *E.faecalis* of treated patients' flora were detected. It seems genes expression alterations, caused by six weeks immunotherapy in patients, which may increase the ability of *E.faecalis* to infection, especially in prostate tissue.

Table 4: Gene expression ratio and *P-values* of *E.faecalis* microflora genes in after immunotherapy treatments vs. related normal subjects

Gene	P-values	Mean ratio
gelE	0.003	1.77±0.14
asa1	0.003	1.68±0.17
Esp	0.003	1.76±0.22
aggA	0.001	1.92±0.27
cylA	0.004	1.74±0.21
cylB	0.003	1.78±0.16
cylM	0.23	1.13±0.05
Eep	0. 04	1.28±0.13
efaA	0.002	1.83±0.19
enlA	0.002	1.94±0.18

Functions of over expressed genes illustrate a pattern of pathogenicity. *gelE* (gelatinase E) is an enzyme with extracellular metalloendopeptidase function which play an important role in virulence and spreading of bacteria in host's body. *asa1* is coding an important factor in aggregation of substances. *esp* (enterococcal surface protein) is involved in evasion of the host immune system by *E.faecalis*. *aggA* functioning in aggregation of substance involved in adherence to mammalian cells and conjugation. *cylA* activate the cytolysin (haemolysin/bacteriocin); *cylB* transport the cytolysin; *cylM* is involved in post-translation modification of cytolysin; *eep* (enhanced expression of pheromone), *efaA* is involved in cell wall adhesin; *enlA* (enterolysin A) belongs to class III bacteriocins which is well-known with cell wall-degrading enzymes produced by Gram-positive bacteria (Bittencourt de Marques and Suzart, 2004, Dong et al., 2015).

The question that come riddle to mind is that how cancer immunotherapy could effect on micro flora. It is known that cancer weaken immune system of patient and immunotherapy try to specifically increase the immune response and activation of humoral and cellular immune response as well. It is possible to presume that these over activation of immune response could kill micro flora and set up a natural selection in microflora population like what antibiotics effect do. This natural selection kills many normal strains of a species and remains stronger strain that could survive against humoral immunity cells. These stronger strains could proliferate faster, lyse proteins and other macromolecules, like gelatin and also construct biofilm. These properties could lead the bacterial strain to pathogenicity in subject (Nowak et al., 2002). Also correlation of several genes that were over expressed in patients after treatment, including *gel E* and *esp*, with antibiotic resistance had been approved (d'Azevedo et al., 2006). It means that if *E.faecalis* of micro flora became virulent, the infections could be resistant to antibiotic treatments; ciprofloxacin resistance, which was clinically observed in 23 patients with prostatitis in present study, may support it.

Majority of cancer therapy methods like radiotherapy and chemotherapy may changes regulation of several mechanisms and pathways of body, which is not directly related or targeted by that method. While normal micro flora has important roles in several vital mechanisms of human, it seems that effects of cancer treatment methods in microflora are strongly ignored (Sullivan et al., 2001).

There are several reports about bacterial and viral infection of cancer patients after treatment by radiotherapy and chemotherapy that mostly lay to cancer care methods but could be related to the changes in number and diversity of microflora as well (Nicolatou-Galitis et al., 2006), (Fijlstra et al., 2015).

Present study is one of the first reports about effects of DC-based cancer vaccines as an important type of immunotherapy on virulence ability of microbial flora in prostate cancer patients. Suggestion of this study is not criticism of immunotherapy for cancer. The point is that while immunotherapy become most used method for cancer therapy, prediction and prevention of side effects of this method is mandatory. In this case, an antibiotic therapy treatment, during or right after end up the immunotherapy period could prevent future infections.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

AUTHORS CONTRIBUTION

Soha Sadeghi, Tayebe Talebzade and Arvin Haghighatfard conceived of the presented idea and they had participatedin study design and performed the statistical analysis. Donya Altafi and Hamed Hojatian participated in laboratory and clinical data gathering and analysis. Arvin Haghighatfard encouraged Soha Sadeghi and Tayebe Talebzade to investigate [a specific aspect] and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript. Soha Sadeghi, Tayebe Talebzade and Arvin Haghighatfard carried out the experiment. Soha Sadeghi and Arvin Haghighatfard wrote the manuscript with support from Sahel Towfigh Rafiei, Shabnam naderifar, Niloofar Ahmadi, Ali dezhgir Donya Altafi and Hamed Hojatian. Sample was provided by Arvin Haghighatfard. Sahel Towfigh Rafiei, Ali dezhgir, Shabnam Naderifar and Niloofar Ahmadi helped supervise the project. Soha Sadeghi and Arvin Haghighatfard supervised the project. All authors give final approval of the version to be submitted and any revised version as well as all authors participate in drafting the article or revising it critically for important intellectual content.

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