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ORIGINAL ARTICLE

Appraising the Increased Expression of p21and p53 Genes in Paraffin-embedded Biopsy tissues of Patients with Bladder Cancer exposed to Mitomycin C

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ABSTRACT

Bladder cancer arises from the epithelial lining layer of the urinary bladder. Understanding the molecular pathway of that can provide some useful information's about therapeutic manners. Hyper activity of P21and p53 genes has been reported in recent years. The purpose of this study was to assess the increased expression of p21 gene in paraffin-embedded blocks of patients with bladder cancer before and after exposure to mitomycin. In this study, 30patients and 5 healthy samples were selected and the blocks of their paraffin-embedded samples were prepared before and after exposing to mitomycin. After sectioning and paraffin removal, RNA was extracted and then cDNA synthesis was performed by using MMULV enzyme, Oligo dt and random hexamer primers. Real Time PCR were performed for evaluating the increased expression ofp21 gene level in samples exposed with mitomycin C. Real Time PCR confirmed the increased expression of p21 and p53 genes in cancer samples which were exposed to mitomycin C. The results showed the lower expression of these genes in patient with bladder cancer and also the expression level of these genes were increased in patients which were exposed by mitomycin C. Investigating on increased expression of these genes in treated patients,can be considered as a proper manner in researches on bladder cancer.

Keyword: *bladder cancer,p21, P53, Real Time PCR, mitomycin C.*

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INTRODUCTION

Bladder cancer is one of several cancers which arises from epithelial lining of the urinary bladder. Rarely the bladder is involved by non-epithelial cancers, such as lymphoma or sarcoma, but these are not ordinarily included in the colloquial term bladder cancer. It is a disease in which abnormal cells multiply without control in the bladder [1,2].Bladder cancer is the 9th leading causes of cancer with 430,000 new cases[3] and 165,000 deaths occurred in 2012[4].tumor suppressor genes have a substantial role in prevention of cancer occurrence. p21 and p53 are one of the most genes that can control the homeostasis by expressing their specific proteins. p21 is a potent cyclin-dependent kinase inhibitor (CKI). The p21 (CIP1/WAF1) protein inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes, and thus functions as a regulator of cycle progression at G_1 and S phases[5]. In addition to growth arrest, p21 can

mediate cellular senescence. And also, Mutations on p53 gene are common in bladder cancer and the Alterations in the p53 pathway contribute to bladder tumor progression and are likely to provide relevant prognostic information to assist in the management of bladder cancer patients [6-8]. Utilization of Chemotherapy drugs are really common in treatment of cancers. Because they can affect the DNA of cancerous cells and prevent these cells from their unregulated function [9], Mitomycin is a really common chemotherapeutic drug in curing bladder cancer. The mitomycins are a family of aziridine-containing natural products isolated from *Streptomyces caespitosus* or *Streptomyces lavendulae* [10]. One of these compounds, mitomycin C, finds use as a chemotherapeutic agent by virtue of its antitumor activity. It is given intravenously to treat upper gastro-intestinal cancers (e.g. esophageal carcinoma), anal cancers, and breast cancers, as well as by bladder instillation for superficial bladder tumors[11-14].Mitomycin C has also been used topically rather than intravenously in several areas. The first is cancers, particularly bladder cancers and intraperitoneal tumors. Because of the importance of this issue, there were several scientists who worked on different aspects of bladder cancer. For example Martin-Doyle et al in 2015 published an article with the title of molecular biology of bladder cancer[15], BA Imnam et al in 2014 worked on a pilot clinical trial of mitomycin-c in non-muscle invasive bladder cancer and also Tolley and his colleagues in 1996 assessed the Effect of Intravesical Mitomycin C on Recurrence of Newly Diagnosed Superficial Bladder Cancer[16]. According to the importance of evaluating the different markers in prevention and the treatment courses, we determined to design a test for working on paraffin-embedded samples which were obtained from biopsy samples and it can be effective in future statistically studies on this issue. So the purpose of this study is to assess the increased expression of p21 and p53 genes in paraffin-embedded biopsy tissues of patients with bladder cancer who have been exposed to mitomycin C.

MATERIALS AND METHODS

RNA extraction by RNX-PLUS solution: RNA extraction was performed by the protocol of Sinaclon Company.

cDNA Synthesis:

Vivantis kit (2 step RT-PCR kit) was used for cDNA synthesis. cDNA synthesis was performed according to kit instructions.

Optimization of Real Time PCR essential factors for B-ACTIN and P21 and p53:

B-actin gene was used as an internal control. After preparing the sequences of B-actin, p21 and p53 on NCBI, the gene specific primers were designed by primer express software. In order to verify the accuracy and specialization of primers, their sequences were blasted. Sequencing of primers is listed in :

Table 1: characteristics of primers:

Name	Sequences	Molecular weight
P21 F	5′-GCAGACCAGCATGACAGATTT-3′	70bp
P21 R	5′-GGATTAGGGCTTCCTCTTGGA-3′	
B-ACTIN F	5′-CGTCTTCCCCTCCATCG-3′	94 bp
B-ACTIN R	5′-CTCGTTAATGTCACGCAC-3′	
P53 F	5′-CCCAGCCCCCTAGCAGAGAC-3′	98 bp
P 53 R	5′-GGTCCCAGCCCAACCCTTGT-3′	

For this purpose, separated reactions were prepared for internal control gene and designed primers in final volume of 20 ml. reactions were performed in parallel on ABI 7500 instrument. Each reaction contained SYBR TM premix (1X), 0.4 mM of forward and reverse primers and 2 µg of CDNA template. The temperature of reaction included 40 complete cycles on 95°c for 15 seconds and 60°c for 1 minute. Dissociation curve analysis was used in order to verify the amplified fragment and absence of non-specific amplification, primer-dimer and pollution. After the reaction, raw data were obtained from device as a CT. and the measurement of gene expression was performed by using ρρCt. gene expression profile was plotted using Graph pad software.

RESULTS

Results of Real time PCR: The Results related to light absorbance of extracted RNA were approved by spectrophotometer. After amplification of CDNA, total number of 30 patients and 5 normal tissue samples were performed by Real Time PCR. Normal samples were used as sample references for comparing the

changes between groups. In order to evaluate the changes in expressions, table 2 and 3 were designed to show the CT-value of the genes before and after using mytomycin C in samples. And also the diagram 1 shows the changes in the expression of examined genes.

GENE	HKG-before treatment	HKG-after treatment	Gene-before treatment	Gene-after treatment	Expression (%)
P21	23.48	23.53	27.53	24.38	3.2
P53	22.90	23.01	26.76	24.64	2.4

Table2: This table contains the CT value of housekeeping genes, p21 and p53 genes in patients and also the changes of their CT value after using mitomycin C.

GENE	HKG-normal	HKG-patient after treatment	Gene-normal	Gene-patient after treatment
P21	23.32	23.53	23.58	24.38
P53	24.87	24.01	23.92	24.64

Table3: This table contains the CT value of housekeeping genes, p21 and p53 genes in normal samples and also the changes of their CT value after using mitomycin C.

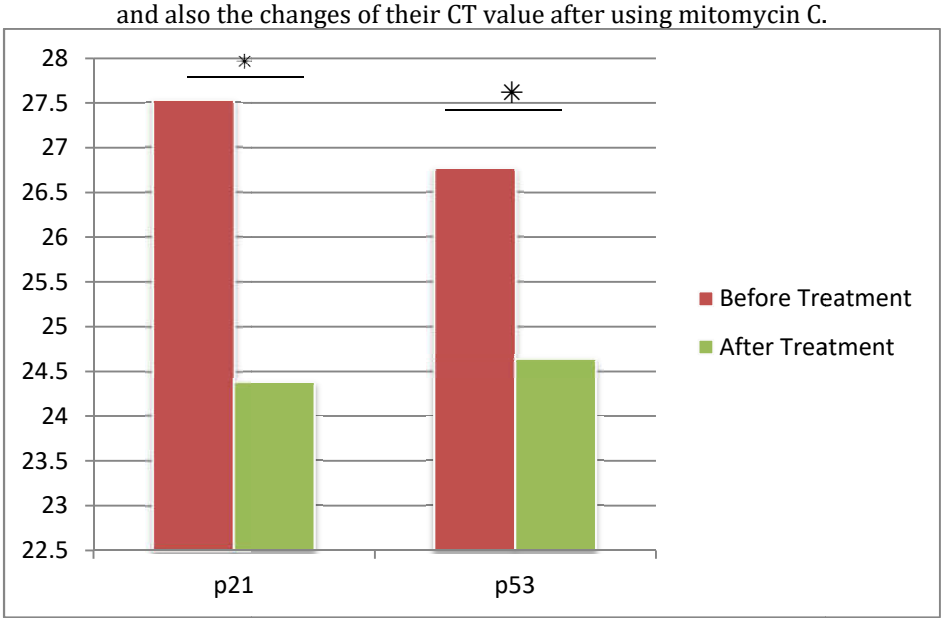

Diagram1: This diagram shows the changes in CT value of p21 and p53 genes in patient samples after using mitomycin C.

As it was shown in table 2, there is no significant difference in CT of B-ACTIN and it shows that the test was performed correctly. And also the CT of P21 and P53 was decreased after utilization of mitomycin c as a chemotherapy drug. Table 3 shows that the expression level of housekeeping genes in normal samples were higher than treated samples and also it was same for mentioned genes. And also Diagram1 shows the information's of table 1 in another way.

DISCUSSION

Bladder cancer (BC) is the most prevalent malignancy in genitourinary system. This malignancy is usually discovered in older patients and the median age at the time of diagnosis is 69 for men and 71 for women [17]. The incidence of BC seems to be moderate in Iran [18].Based on the reports presented in 2005 by the Cancer Office at the Non-communicable Deputy of the Iranian Center for Disease Control and Prevention, BC accounts for 7.04% of all cancers in Iran. According to this report, the age-specific incidence rate of BC in Iran is 11.30 in males versus 2.86 in females in a population of 100,000. Surprisingly, in some areas of Iran the incidence of this cancer reaches to as high as 15.9 in a population of 100,000 [19]. The function of p21 is related to cell apoptosis, progression and malignancies. It is thought that p21 and p53 are related to the cancer formation but is not related to tumor grade. Different types of treatment are available for patients with bladder cancer [20-22]. Some treatments are standard and some are being tested in clinical trials [23-25]. Chemotherapy is a cancer treatment that uses drugs to stop the growth of cancer cells, either by killing the cells or by stopping them from dividing. When chemotherapy is taken by mouth or injected into a veinor muscle, the drugs enter the bloodstream and

can reach cancer cells throughout the body. When chemotherapy is placed directly into the cerebrospinal fluid, an organ, or a body cavity such as the abdomen, the drugs mainly affect cancer cells in those areas [26-29]. For bladder cancer, regional chemotherapy may be intravesical. The way the chemotherapy is given depends on the type and stage of the cancer being treated. Mitomycin c (MMC) is one of the most used agents with limited side effects. In fact MMC has a high molecular weight and is relatively hydrophobic, resulting in less systemic absorption [30.31.32]. Regimens are based on weekly instillations but despite many studies there is not universal consensus on timing and duration of therapy [33]. MMC early utilization seems to be effective in preventing the tumor recurrence in low risk non muscle invasive neoplasms. Opposite of p53, Brief researches have carried out on evaluating the P21 gene expression level in under chemotherapy patients by using their pathological paraffin-embedded block samples. So the identification and use of new markers in diagnosis and progression of disease and also using them in studying the therapeutic effects of drugs can be considered as a necessary and inevitable point in developing the therapeutic manners. There were many studies on using the molecular markers in in assessing the progression of cancer and also for studying the use of mitomycin in treatment of bladder cancer. Some of them are mentioned below. Granada *et al* in 2005 studied the Single Perivascular Delivery of Mitomycin C Stimulates p21 Expression and Inhibits Neointima Formation in Rat Arteries [34]. In comparison to their study we worked on the enhanced expression of P21 and P53 genes that could be more documented in functionality of mitomycin. Tolley and his colleagues in 1996 assessed the Effect of Intravesical Mitomycin C on Recurrence of Newly Diagnosed Superficial Bladder Cancer [35]. It was a really beneficial study. Because it is truly important to detect cancers in really stages of recurrence and followed them we designed this study to use two substantial tumor suppressor genes to evaluate the increased expression of them after utilizing the mitomycin and it could an effective title for detecting the stages of bladder cancer in further studies. Bohle et al in 2003 compared the Intravesical Bacillus Calmette-Guerin against Mitomycin C for Superficial Bladder Cancer: A Formal Meta-Analysis of Comparative Studies on Recurrence and Toxicity[36]. In their study they compared to ways as therapeutic manners and also they mentioned some beneficial effects of using mitomycin and it could be one of the reasons of choosing mitomycin our study. Solsona et al in 1999 worked on the effectiveness of a single immediate mitomycin C instillation in patients with low risk superficial bladder cancer [37]. One of their remarkable points was their short term and long term follow up, but despite of considering the clinical symptoms, it can be effective to pay more attention to molecular biology and paying attention to molecular markers as a determinant of the stages of cancers. Heney *et al* in 1983 published an article with the title of "Superficial bladder cancer: progression and recurrence"[38]. As we mentioned during the article, it is really important to detect different stages of cancers by evaluating the molecular markers as a monitoring factor. And also we preferred to use the paraffin-embedded blocks; because they can be really useful in statistically long term researches. Stein et al in 1998 studied the effect of p21WAF1/CIP1 expression on tumor progression in bladder cancer [39]. As it was mentioned before, the amount of the expression of P21 increases in higher stages of bladder cancer, and that's because of the changes that happen in molecular pathways. So we preferred to study the p51 increased expression beside the p21 and it could be a confirmatory factor in function of mitomycin. And also Stein with another group in 1998 studied the effect of p21WAF1/CIP1 expression on tumor progression in bladder cancer [40]. As it was mentioned before, the amount of the expression of p21 increases in higher stages of bladder cancer and that's because of the changes that happen in molecular pathways. So we preferred to study the P51 increased expression beside the p21. It could be a confirmatory factor in function of mitomycin C.

CONCLUSION

From the results, it can be summarized that p21and p53 gene expression are diagnostic molecular markers in bladder cancer and by using the paraffin-embedded block as a sample, different strategies can be applied in prognosis, patients treatment, long term monitoring and designing statistical researches, On the other hand it can be mentioned that the expression level of p21 and P53 genes were increased by using mitomycin C. Following the outcomes, more assessments on the amount of different tumor suppressor genes in presence of chemotherapy drugs can be utilized as an effective expectancy in choosing an appropriate treatment strategy.

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