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REVIEW ARTICLE

A Single Nucleotide Polymorphism in the *MMP9* Promoter Affects Lung Cancer and Clinicopathological Properties in Iranian Population

Somayeh Taghvaei^{1*}, Leila Saremi² and Majid Motovali-bashi³

¹National Institute of Genetic Engineering and Biotechnology, Department of Medical Biotechnology, Tehran, Iran

²Science and Research Branch, Islamic Azad University, Department of Biology, Tehran, Iran

³Department of Biology, Faculty of Sciences. University of Isfahan, Isfahan, Iran

ABSTRACT

Background: Lung cancer is the most common cancer with 2,206,771 new cases in 2020 in worldwide. *MMP9* is a member of matrix metalloproteinase family that is also known as gelatinase B or IV type collagenase (92KD). *MMP9* through degrading of Extracellular Matrix (ECM) and releasing of growth factors has fundamental role in the tumorigenesis process. The C -1562 T SNP in the *MMP9* promoter increases *MMP9* expression and susceptibility to lung cancer. Then, the aim of this present case-control study was to investigate whether genetic variations of the *MMP9* gene may constitute markers for lung cancer risk in males and in positive family history people in Iran.

Methods: This is a case-control study including 120 lung cancer patients and 100 healthy controls. Polymorphism in the C -1562 T region was genotyped by PCR-RFLP assay. Odds Ratio (ORs) and 95% Confidence Intervals (CIs) were estimated by chi-square test from comparison of genotypes between lung cancer patients and healthy controls, using SPSS version 26.0. T-test and Image J software was also used.

Results: The distribution of C-1562T genotype was significantly associated with the risk of lung cancer (Odds Ratio [OR] = 2.56, 95% Confidence Interval [CI] = 0.06-23.82). The further stratification analyses shown that males and patients with positive family history may increase risk of lung cancer.

Conclusion: Our results indicated that the *MMP9* C -1562 T polymorphism affects risk of lung cancer. In addition, men with T allele (OR = 3.94, CI = 1.47-10¹.55) and patients with TT genotype and family history (OR = 2.18, CI = 1.03-4.59) exposure to higher risk of lung cancer.

*Corresponding author

Somayeh Taghvaei, National Institute of Genetic Engineering and Biotechnology, Department of Medical Biotechnology, Tehran, Iran

Tel: +98-912-057-4390

ORCID: 0000-0002-4282-1224

E-mail: somayyetaghvaei@gmail.com

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- SNP
- Lung cancer
- Iran

INTRODUCTION

Carcinogenesis is a complex process in which cells undergo genetic and epigenetic changes. Genetic polymorphisms are the internal cause of cancer [1]. Matrix metalloproteinases are multifunctional proteases that proteolyze Extracellular Matrix (ECM) components with subsequent release of bioactive components and proteins [2,3]. They participate in membrane shedding. They play an important role in chemokine processing. They change the mode of activity of other proteases. MMPs is restricted to certain types of cells or tissues under physiological conditions, while the majority are widely expressed and play many essential physiological roles [4,5]. Altered expression and activation of the matrix of metalloproteinases generally stimulate signs of tumor progression, including invasion, angiogenesis, and metastasis, and are associated with short survival in a variety of different cancers [6,7].

Lung Cancer is the third abundant cancer in Iran and the most abundant cancer in the worldwide (Figures 1A and B). This cancer is the most abundant cancer in

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males in Iran and in worldwide (Figures 1C-F). In NSCLC, strong correlations between increased levels of MMPs and decreased survival time have been reported [8]. MMP family is classified into five main classes (collagenases, gelatinases, stromelysins, membrane-type MMPs, and matrilysins) on the basis of their putative substrate specificity, primary structure, and cellular location [5,9,10]. MMPs comprise a structurally and functionally related family of zinc metalloproteinase that is degrading extracellular matrix and basement membrane barriers [11]. Thus, they are play

a key role in angiogenesis, inflammatory processes, cancer development, cell proliferation, and apoptosis [12].

MMP9 gene located on human chromosome 20q 12.2-13.1 [13]. The *MMP9* promoter is in a 2.2 kb flanking region. The *MMP9* promoter contains binding site (s) of transcription factors AP1, SP-1, Nuclear Factor- κ B, ETS, STAT, TGF- β , and also having elements of responding to retinoic acid [14]. A complex of c-Jun/Fra-1 and STAT3 was shown that activate the human *MMP9* promoter [9]. *MMP9* is

Figure 1 A) Number of new cases in 2020 in Iran, B) Number of new cases in worldwide, C) Number of new cases, males in Iran, D) Number of new cases, females in Iran, E) Number of new cases, males in worldwide, F) Number of new cases, females in world wide.

synthesized as zymogen that requires proteolytic cleavage of the amino terminal prodomain (~10KD) to be activated by MMP2, MMP3, and hypochlorous acid. MMP9 has also been able to activated by a plasmin dependent pathway and MMP2 and MMP10 [15]. Endogenous inhibitors such as TIMPs directly regulate the activity of MMP9. Other inhibitors are TFPI2, α 2-macroglobulin, Thrombospondin 1 and 2, and reck protein [16]. MMP9 has cell surface and secreted forms. In contrast to soluble MMP9, membrane-bound MMP9 is substantially resistant to inhibition by TIMPs and may be responsible for the proper action of neutrophils in *in vivo* [17]. MMP9 dimerization with another MMP9 or MMP1 are inhibited when MMP9 attaches to TIMP-1. The activity of other proteases and cytokines that are important in lung diseases that is regulated by MMP9. MMP9 degrades α 1-antitrypsin, protects the elastase activity of neutrophils, and enhances the collagenolytic activity of MMP13 and fibroblasts in collagen gels [14]. MMP9 might increases angiogenesis by activating angiogenic factors, for example TGF- β [18] and increases tumor growth by activating growth factors, for example IGF and TGF- β [19]. TGF- β also results in induction of MMP9, PDGF-A and B by endothelial cells while, expression of TIMPs decreases [20]. Egeblad has shown that matrix metalloproteinases affect tumor cell behavior and play an important role in several stages of cancer progression, consist of immune surveillance, angiogenesis, cell growth regulation, and apoptosis [21].

Decreased HDGF expression inhibits cell migration and invasion in the prostate cancer glass by regulating the mesenchymal-epithelial transmission signaling pathway plus the MMP2 and MMP9 signaling pathway [22]. EGR/EGFR signaling enable to activate PI3K/AKT signaling pathway that by inducing foxO1 nuclear export activate MMP9 to stimulate NSCLC attack. Inhibition of MMP9 attenuates tumor invasion and high expression of MMP9 in serum and no tissue is a risk factor for tumor stage and poor outcome [23]. MMP9 can alter cell function by regulating cytokines and matrix-bound growth factors [14]. High expression of MMP9 and its proteolytic activity is potentially diagnostic marker of several types of cancer [24]. Figure 2 is Kaplan-Meier plot of MMP9 expression in lung cancer through Kaplan-Meier Plotter database. As you can see, higher levels of MMP9 expression are related to poorer prognosis with $p < 0.05$. Figure 3 also represents higher expression of MMP9 in lung cancer samples than control samples. In the normal lung, MMP9 is not produced by resident cells but is produced under some forms of stimulation. Lung cancer cells, both primary and metastatic continuously produce MMP9, which may be associated with the ability to metastasis [14].

Single nucleotide polymorphisms are the most common genetic variation. Single nucleotide polymorphisms can alter the expression and function of encoded proteins. SNPs alter individual susceptibility to common diseases such as cancer, Alzheimer, diabetes, atherosclerosis, and COPD [25,26]. A lot

Figure 2 Kaplan-Meier plot for MMP9 expression in lung cancer.

Figure 3 Boxplot of MMP9 higher expression in lung cancer patients compared with controls with $p < 0.01$ in GEPIA2 database, Red: tumor samples and Blue: controls.

of single nucleotide polymorphisms has been identified in the genes encoding MMPs and TIMPs. There is evidence that C -1562 T rs3918242 Single Nucleotide Polymorphism (SNP) affects MMP9 expression with allele T having promotor activity 1.5 times greater than C allele [27,28].

The association of this polymorphism have been shown with lung cancer susceptibility, tumor progression and malignant phenotype of gastric cancer susceptibility, tumor progression of breast cancer, colorectal risk, HNSCC progression, and salivary gland cancer susceptibility and **no association of this polymorphism** has been shown with PCA development and aggressiveness [24,29-34]. We found a significant association between this polymorphism, lung cancer and clinicopathological properties. The purpose of this study was investigation the relation between the C -1562 T MMP9 polymorphism and risk of lung cancer and clinicopathological factors including gender and family history.

MATERIALS AND METHODS

Study population

This was a hospital-based case-control study which included 120 patients (75 males and 45 females: rang of age at diagnosis: 29–70 years) and 100 controls (45 males and 55 females: rang of age at diagnosis: 26–76 years). All patients were diagnosed from the research center of tuberculosis and lung diseases from Iran.

Data collection

Information of potential risk factors of lung cancer was collected personally through computer-assisted questionnaires during the first hospital admission at diagnosis time. Questionnaires collected information of gender and family history.

DNA extraction

Five milliliters of venous blood from each subject was drawn in vacutainer tubes containing EDTA and was stored at -20°C . Genomic DNA was extracted by using proteinase k digestion followed by a salting out procedure according to the method published by miller [35] (Figure 4).

Genotyping of *MMP9* polymorphism

The C-1562 T genotype was determined using PCR-RFLP assay. The primers that were used including: The forward: 5'-ATATAAAATGCCTGGCACATAGTA3' and reverse: 5'CTCCCTCACTCCTTTCTTC-3'. PCR reactions were performed in 25 μl reaction volume containing 150 ng of genomic DNA: 2.5 μl 10X PCR buffer, 20 pmol of each primer, 0.5 μl 10 mM dNTP, 0.75 μl 50 mM MgCl₂, and 0.3 μl Taq DNA polymerase (Cinnagen, Iran) (Figure 5). The PCR cycle conditions consisted of an initial denaturation step for 3 min at 95°C that followed by 30 cycles of 30s at 94°C , 30s at 65°C annealing step, 1 min at 72°C elongation step and subsequently a final elongation at 72°C for 10 min. The PCR products were separated by electrophoresis in 1% agarose gel and subsequently stained with ethidium bromide. Then the PCR products were digested with Sph1 (Metabion) and genotypes were defined according to distinct band patterns including: CC (460 bp), TT (202 and 258 bp), CT (202, 258, and 460 bp) (Figure 6). A 5 μl aliquot of the PCR product was digested overnight at 37°C in an aliquot with 1 μl 10 X Tango TM (enzyme buffer) and 2.5 u Sph1. The digested fragments were separated by electrophoresis with 2% agarose gel then in order to verify digestion, the digested bands were analyzed by ImageJ software. Figure 6 displays genotype TT with two bands 202, 258 bp had two peaks, but genotype CC had one peak with 460 bp, which is equal to the sum of two peaks TT. A random 20% retyped that yielding identical results. The association of the genotype with categorical variable also was evaluated by χ^2 test.

Figure 4 Electrophoresis of DNA genomic of case and control peoples. Numbers of I, II, III of patients with lung cancer and numbers IV, V, VI, VII of controls peoples, M: 100 bp DNA Marker.

Figure 5 PCR of various peoples of cancer and control peoples and survey on 1% agarose gel.

Figure 6 The PCR-RFLP analysis of the -1562 polymorphism of five patients with lung cancer. The RFLP products were separated with 2% agarose gel. Numbers above the panel are case numbers. Genotypes are indicated below each case. CC: positive homozygote, CT: heterozygote, TT: negative homozygote.

Statistical analysis

Statistical analyses were performed using SPSS 26.0 software package. Hardy-Weinberg analysis was performed via comparing the observed and the expected genotype Frequencies using chi-square test. The distribution of the *MMP9* genotype in cancer patients and healthy controls was compared by chi-square test. Statistical significance was set at $p < 0.05$. The mean age calculated by T-test. The patients were divided to the people with family history and the people without family history. The relationship between

Figure 7 Results of investigation of digested (TT genotype) and non-digested (CC genotype) products.

MMP9 polymorphism and gender and family history were calculated. The Odds Ratio (ORs) and 95% Confidence Interval (CI) showed relation of gender and family history with the genotypes of lung cancer.

RESULTS

This study was a hospital-based case-control study which included 120 patients (45 males, 55 females) and 100 controls (75 males, 45 females). We examined the C -1562 T polymorphism of MMP9 gene promoter by PCR-RFLP in lung cancer patients and controls (Figure 5) and verified digestion PCR-RFLP products by Image J software (Figure 7). The relevant characteristics of studied subjects were shown in table 1. Distribution of the C -1562 T polymorphism genotypes in lung cancer patients and controls were shown in table 2. Genotypes were in hardy-Weinberg equilibrium. There were statistically significant differences between the cases and the controls in terms of family history and gender distributions $p = 0.000$ and $p = 0.009$ respectively (Table 1).

The genotype frequencies were significantly different between the cases and the controls ($p = 0.028$), with the -1562 TT genotype that being overrepresented in the cases.

Analyses showed the -1562 TT genotype carriers had a 2.56-fold elevated risk for the development of lung cancer (adjusted OR = 2.56, 95% CI = 0.06-23.82) compared with the non-carriers. The TT+CT genotype carriers were also at a 2.31 increased risk of developing the cancer compared with the CC genotype carriers (adjusted OR = 2.31, 95% CI = 0.13-14.23) (Table 2).

Gender groups analysis was shown that male with the CT+TT and TT genotypes have higher risk for lung cancer (OR = 3.94, CI = 1.47-10.55) and (OR = 2.27, CI = 1.04-4.95), respectively (Table 3). We divided cancer people to two groups: positive family history and negative family history. Our results showed increasing risk of lung cancer in people with positive family history (OR = 2.18, CI = 1.03-4.59) (Table 3).

DISCUSSION

The ECM is now known to contain growth factors, their binding proteins and other bioactive molecules. Some of them are revealed only after proteolysis. The ECM transmits essential information to lung cells, organizes their differentiation, and regulates their functions. Lung cells also are able to reciprocate with the regulation of production of ECM [36]. Regulated degradation of ECM is an important property in the several of physiological and pathological processes, such as tissue remodeling, embryonic development, tissue repair and growth, invasion, and metastasis of cancers [13,37].

In addition to degradation of extracellular matrix and basement membranes, MMPs can also cleave molecules of cell surface including the tumor suppressor E-cadherin. MMP9 able to releasing growth factors for example bFGF, and Insulin Growth Factor (IGF) and MMP9 is also an essential enzyme in tumor growth and angiogenesis [38,39]. The studies also have been demonstrated a critical role for MMP9 in releasing of the key angiogenic factor VEGF from the ECM, thereby stimulating angiogenesis that causes to tumor growth [8]. Processing of ECM components by MMPs yields bioactive components. For example, MMP2 and MMP9 expose a hidden epitope within type IV collagen that

Table 1: Controls and cases properties.

Clinical properties	Controls		Cases		p value*
	n	%	n	%	
Gender					
Male	45	(45%)	75	(62.5%)	0.009
Female	55	(55%)	45	(37.5%)	
Family history					
Having family history			68	(56.67%)	
Not having family history			52	(43.33%)	
N: all of the people.					
*p value was calculated with X ² -test					

Table 2: Genotypes and allele frequency of *MMP9* and risk of lung cancer.

Genotypes	Controls		Cases		p value*	OR (95% CI)
	n	%	n	%		
CC	29	(29%)	18	(15%)	0.002	2.56(0.06-23.82)
CT	48	(48%)	50	(41.67%)		
TT	23	(23%)	52	(43.33%)		
CT + TT	71	(71%)	102	(85%)	0.012	2.31(0.13-14.23)
C allele	106	(53%)	86	(35.84%)	0.000	2.01(1.37-2.96)
T allele	94	(47%)	154	(64.16%)		

*p value was calculated with X²-test.

Table 3: Relationship of *MMP9* C -1562 T genotypes with clinicopathological properties of lung cancer patients.

Clinical properties	Controls (CC CT TT)			Cases (CC CT TT)			OR (95%CI) (CT + TT ^a TT ^b)	
Gender								
Male	13	19	13	7	32	36	3.94(1.47-10.55)	2.27(1.04-4.95)
Female	16	29	10	11	18	16	*p = 0.6	2.48(1-6.12)
Family history								
Having family history				13	55	35	*p = 0.14	2.18(1.03-4.59)
Not having family history				5	47	17		

*No relation.

In all of cases that the positive relation is mentioned, always $P < 0.05$ is being, a) OR calculated for CT+TT against TT, b) OR calculated for TT against CC + CT.

stimulates angiogenesis, while anti-angiogenic factors such as endostatin can form type XVIII collagen [4].

Binding of *MMP9* to ECM components activates these enzymes due to accelerated automatic activation or activation without removal of pro-sequence of these enzymes. Hence, *MMP9* may contribute in multiple ways in all stages of cancer development including initiation, progression, and metastasis [15].

In 1999, Zhang showed that SNP in *MMP9* promoter could affect transcription activity. This substitution makes a sequence for ETS transcription factors and inhibits binding of inhibitory transcription factors [40]. Allele that results in upper expression of *MMP9* may facilitate initiation of lung cancer through digesting of ECM, and releasing of growth factors and susceptibility to lung cancer may increase through modification of the environment or regulation of apoptosis and cell proliferation.

Lung cancer is the most common cause of cancer deaths in world-wide. In Iran in 2020, 10 465 people have been infected to lung cancer. High costs for control and treatment of lung cancer and high mortality results of lung cancer development, significance of diagnostic methods for early onset lung cancer shows. According to studies conducted by different researchers the presence of the T allele results in increased activity and increased expression of the putative promoter of *MMP9* [41]. In our study in Iran's population, the share of the T allele in lung cancer patients was more than controls. These findings suggest that the individuals

carrying of the T allele of the genetically are susceptible to lung cancer development.

According to the mentioned studies, this can be argued that T allele of the *MMP9* promoter activity results in increasing of *MMP9* protein that increases degradation of extracellular matrix, releasing of growth factors, and ease the process that can start and spread lung cancer [38,42].

Differences in risk estimates for TT homozygous than heterozygous for this allele that may also be due to gene dosage effects [43]. In fact, in the presence of environmental signals and transcription factors, transcription of T two allele much more than a T allele thus, produced *MMP9* proteins of the individuals TT homozygous for this allele more increases compared with heterozygous [40]. One of the reasons for the different expression of MMP enzymes in the various tissues and the tumors can be the function of ETS factors. ETS transcription factors in cooperation with other regulatory transcription factors can cause that your partner proteins activate transcription of various genes or inhibit them. Therefore, this possibility exists that their partner proteins are different in a variety of diverse cells and hence the expression of ETS-dependent of *MMP9* promoter can be different in the types of cells. On the other hand it is possible that other pathways independent of the promoter SNP at position -1562 control the expression of *MMP9* [44].

Regulation of transcription is a complex process that often take places in the context of special factors. This is possible that transcriptional regulation of *MMP9*, directly in the presence of ETS connecting sites in the T SNP has not

increase. It offers that the gene expression is the second compared to the SNP. This means that tissue specific and various disease conditions can have different functions [45]. Genetic of diseases and histological differences can also affect the expression of *MMP9*. For example, P53 destroys relationship between DNA and the AP1 activator of the transcription complex and inhibits the *MMP9* expression. Then, when the P53 mutates and its inhibitory action on the *MMP9* expression lost, the *MMP9* expression in these tumors increases thus, T polymorphism in this study can be further increases expression of the *MMP9* [46].

In this study *MMP9* promoter polymorphism in case-control study was compared and results by the OR parameters with 95% confidence interval were presented. The odds ratio is the way for comparing of groups. The T allele was detected more frequently in patients (OR = 2.01, CI = 1.37-2.96). *MMP9* substitution has been shown to upregulate the promoter activity. Thus, the presence of the -1562 T *MMP9* allele associates with decreased capacity of a putative transcription repressor protein that bind to the promoter region, with subsequent increase in gene expression [10].

Johanna, et al. [47] showed Expression of MMP-2 and *MMP9* in breast cancer looks to be partly associated to expression of AP-2 and HER2. Sook park, et al. [31] exhibited *MMP9* level relates to susceptibility of colorectal cancer. Hajihoseini, et al. [33] also demonstrated TT genotype is more in HNSCC cases than controls. Motovali-bashi, et al. [48] suggested serum *MMP9* level is a potential diagnostic marker for predicting breast cancer occurrence and progression. Padala, et al. [49] showed synergistic effect of *MMP9* -1562 C/T, *MMP1* -1607 1G/2G, and *MMP3* -1171 5A/6A on breast cancer. Hemati, et al. [50] concluded higher plasma *MMP9* level in breast cancer patients with *MMP9* T allele. Owyang, et al. [51] represented *MMP9* is an important component of the metastatic niche early in carcinogenesis and increase colonizing circulating tumor cells to the lungs. Radunovic, et al. [34] concluded carriers of CT genotype had roughly a 2-fold raise in risk for salivary gland cancer compared to wild type homozygotes (CC) ($p = 0.02$). In the other study in south Iran, TT genotype showed strong relation with lung cancer initiation and progression $\chi^2 = 13$, $p = 0.01$ [24]. Matsumura, et al. [29] also indicated that the T allele in the *MMP9* promoter is linked to the invasive phenotype of gastric cancer.

Wieczorek, et al. [52] demonstrated the combined genotype *MMP2* C -1306 T allele T with *MMP9* C -1562 T allele T increases bladder cancer risk. Zhang, et al. [53] observed *MMP9* C -1562 T polymorphism may diminish the colorectal and lung cancer risk. Zhou, et al. [54] performed a meta-analysis and indicated no association -1562 *MMP9* polymorphism and breast cancer in Chinese population. Tao, et al. [55] found no association between -1562 polymorphism and bladder cancer in Chinese population. Peng, et al. [56] reported *MMP9* C -1562 T

polymorphism may not be a major susceptibility factor for most cancer types. Bayramoglu, et al. [57] found CC genotype is remarkably more in lung cancer patients than controls in Turkish population. Yan, et al. [58] found no associations between *MMP9* C -1562 T polymorphism and bladder cancer susceptibility. The controversial results can due differences in subtypes of cancer, sample size, ethnicity, environmental factors and gene-gene interactions.

Moreover, the risk of lung cancer significantly has been increased in males with *MMP* polymorphisms [59-61]. Since the ovarian steroids are decreasing the *MMPs* expression. Therefore, it expects that women compared with men have lower levels of *MMP9* protein. The levels of estrogen in the regulation of the *MMP9* expression can be even more important than the function of polymorphism [62]. Therefore, we can conclude that T allele carrier's males have higher risk of lung cancer than T allele carrier's women. We found T allele is more in males.

In this study, the positive family history of cancer with T polymorphism compared to the negative family history of cancer. In the study by Rahimi, et al. [63] the frequency of *MMP9* CT + TT genotype was higher in the patients with a family history of cancer in first degree-relatives [CT + TT, (36.8%), CC, (63.2%)] than those without a family history of cancer [CT + TT, (28.3%), CC, (71.7%), $p = 0.37$].

Okada, et al. [64] reported also the haplotype *MMP9*-1562C/279Q/668Q was more mostly saw in individuals with a history gastric cancer and in individuals with a history gastric cancer in one parent and sibling(s) compared with those without family history (OR 3.35 and OR 3.51, respectively).

We acknowledge several limitations to our study: firstly, this is a hospital-based case-control study. Secondly, we only evaluated the *MMP9* -1562 polymorphism that has been suggested to be associated with *MMP9* expression levels which may be one reason why we observed a stronger association for the CT + TT genotypes than the TT genotype in most of the comparisons. Then, interactions of gene-environment and gene-gene were not determined in our meta-analysis. Thirdly, the potent proteolytic activities of *MMPs* are mainly regulated by the balance with specific tissue inhibitors of *MMPs*. However, adding other polymorphism into the analysis will require more large-scale and high-quality studies in future.

CONCLUSION

In summary, our data provide the molecular epidemiological evidence of the *MMP9* promoter polymorphism associated with the risk of lung cancer in men and in people with positive family history in Iranian population. Our observations provide direct evidences of role of *MMPs* in altering cellular microenvironment, thereby facilitating initiation and progression of tumor.

These finding may ultimately help in identifying high-risk populations. Further study of the biological ramifications of this polymorphism may add to our understanding of the biology of the disease and provide potential foci for targeted therapies.

STATEMENTS AND DECLARATIONS

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Data availability statement

All data generated or analyzed during this study are included during article

Ethic approval

Ethic code IR.UI.REC.1400.018 was issued by university of Isfahan.

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