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RESEARCH

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Investigating the importance of EGFR (– 216G/T), Exo1 (K589E) and LEP (– 2548G/A) gene polymorphisms with risk of lung cancer as potential diagnostic biomarker in Iranian population

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Abstract

Background In Iran, lung cancer is the third most common type of cancer and its prevalence is increasing rapidly. Identification informative genetic polymorphisms in cancer causing genes including epidermal growth factor receptor (EGFR) as key gene in control of cellular proliferation via intrinsic tyrosine/kinase activity, exonuclease 1 (EXO1) as one of the upregulated gene in different human malignancies and leptin (LEP) participate in carcinogenesis in lung cancer appears to be used as potential genetic markers for predicting lung cancer risk. There is no study about investigate association of the *EGFR* (– 216G/T), *Exo1* (K589E) and *LEP* (– 2548G/A) gene polymorphisms with risk of lung cancer in Iranian population. The aim of this study was investigating the association of *EGFR* (– 216G/T), *Exo1* (K589E) and *LEP* (– 2548G/A) gene polymorphisms with risk of lung cancer as a potentially diagnostic biomarker in Iranian population.

Methods In this case–control study, A total of 100 patients with lung cancer and 100 age and gender-matched healthy controls were recruited into this study and the association between EGFR (– 216G/T), Exo1 (K589E) and LEP (– 2548G/A) gene polymorphisms with the risk of lung cancer was investigated by using PCR–RFLP technique and bioinformatics approach.

Results The rs712829 of EGFR gene show that a significant statistical difference between G allele and risk of lung cancer ($P=0.001$, OR = 2.976, CI = 95%, 1.557–5.691), in contrast, the T allele and TT genotype show a protective role against the risk of lung cancer. Result of in silico analysis indicated that the rs712829 alter splicing and promoter regulation of EGFR gene and associated with the risk of lung cancer. PCR–RFLP result for rs1047840 of *Exo1* gene showed that the AA genotype and A allele of this polymorphism associated with risk of lung cancer, whereas the GG genotype show a protective effect against the risk of lung cancer ($P=0.004$, OR = 5.391, CI = 95%, 1.690–17.200). On the other hand, in silico analysis showed that the existence of rs1047840 in *Exo1* gene influence lung cancer susceptibility. For rs7799039 of LEP gene, PCR–RFLP analysis showed that, there is no significant association between this polymorphism and the risk of lung cancer.

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Conclusion The rs712829 of *EGFR* gene and rs1047840 of *Exo1* are associated with risk of lung cancer among Iranian population and can be used as a potentially candidate biomarker for early detection and primary prevention.

Keywords Lung cancer, Early genetically detection, SNPs, *EGFR*, *Exo1*, *LEP*

Background

Lung cancer (LC) is the cancer with the highest incidence and mortality rate in the over world [1, 2]. LC is the most common cancer that usually diagnosed in an unresectable and advanced stage [3]. Most patients diagnosed with LC presented with distant or metastatic disease; less than 20% were diagnosed with localized [4].

In 2018, 2.09 million people were diagnosed with LC and there were 1.76 million deaths from LC [5]. LC is the third most common type of cancer in Iran and its prevalence is increasing rapidly [6]. LC has been classified into two major categories: first group is non-small cell lung cancer (NSCLC), which collectively comprises adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Second group is small cell lung cancer (SCLC), which is more aggressive and has worse survival rates. Approximately 85–87% of lung cancers are NSCLC [3–5, 7]. The etiology of LC was unclear, but the development of LC is known to be multifactorial. Genetic and environmental factors such as addiction to tobacco use, radiation therapy, other lung diseases, race and family history of LC play an important role in disease susceptibility [4, 8, 9]. Rahal et al. study indicated that genetics factor such as single nucleotide polymorphisms (SNPs) may play a role in risk of LC [10].

The finding of genetic markers associated with increase susceptibility to LC is an active area of research [11]. Several reports in different populations have been published suggesting that SNPs of the EGFR (− 216G/T), Exo1 (K589E) and LEP (− 2548G/A) genes are associated with LC risk [12–14]. The fact that numerous histological types of lung cancer responded to medications that inhibit EGFR and/or its downstream effectors demonstrates the function of EGFR in mediating lung cancer etiology. These observations imply the possibility of lung cancer-causing mutations and/or polymorphisms in the EGFR gene. Epidermal growth factor receptor (EGFR), a trans-membrane glycoprotein with tyrosine kinase activity that encoded by a gene located on 7p12 and is a main regulator of different signaling pathways and it belongs to the ErbB family and plays a significant role in regulating many different signaling pathways that include cell proliferation [15, 16]. *EGFR* is frequently overexpressed in many cancers including LC [12]. Among variants of *EGFR* gene, − 216G/T (rs712829), a functional polymorphism in the *EGFR* promoter, the replacement of G by T at position − 216 increases promoter activity by 30%, thereby resulting in a higher *EGFR* expression level and causes carcinogenesis [15, 17]. In addition, Bashir et al. has reported the association of − 216G/T polymorphism of EGFR gene with the risk of LC in different populations [12]. Another gene polymorphism that involved in the development of LC is the *Exo1* gene. Exonuclease 1 (*Exo1*) located on human chromosome 1q42-q43 and encoding an 846 amino acid protein that play important role in DNA repair by MMR mismatch repair pathway [18, 19].

Many researches show that there is an association between *Exo1* inactivation and increased likelihood of tumorigenesis in lung tissue [19, 20]. Particularly in patients with a history of smoking, a number of EXO1 SNPs have been indicated to be associated with an increased susceptibility to lung cancer and suggest a potential oncogenic role for EXO1. Among SNPs of *Exo1* gene, the rs1047840 (K589E) is most important variant associated with the risk of various cancers, including breast cancer, gastric cancer, oral cancer and LC [13, 19, 21–23]. The rs1047840 affects *Exo1* mRNA expression and cause inefficiency in the DNA repair process as a result can led to LC [13, 24, 25].

Recent studies have showed that leptin is crucial to the development of tumors [26–28]. Leptin (LEP) is necessary for adipocyte homeostasis, and leptin resistance causes adipotoxicity and cellular fat death, which raise the risk of neoplasia [29]. LEP located at chromosome 7q31.3, encodes a 16 kDa protein that to be associated with endocrinologic metabolism [30]. *LEP* participates in the regulations of energy balance, adiposity, endocrine systems, immunity, angiogenesis and oncogenesis [31, 32]. Several studies indicated that LEP − 2548 G/A (rs7799039) are correlated with the development of many cancers such as oral cancer [33], prostate cancer [34], oropharyngeal cancer [35], breast cancer [36], hepatocellular carcinoma [37] and LC [14, 38].

The existence of − 2548 G/A polymorphism in LEP gene associated with high gene expression and twofold LEP secretion. Higher mRNA expression of Leptin may increase risk of LC [14]. The prior reports representing different ethnic population have presented controversial results about studied SNPs. Identification of genetic variants involved in LC can be used as clinical biomarkers for early genetically detection [39]. The use of these clinical biomarkers can play an important role in reducing mortality and also reducing material and spiritual

damages caused by this disease. The aim of this study was investigating the association of EGFR (− 216G/T), Exo1 (K589E) and LEP (− 2548G/A) gene polymorphisms with risk of lung cancer as a diagnostic biomarker in Iranian population. The use of these clinical biomarkers can play an important role in reducing mortality and also reducing material and spiritual damages caused by lung cancer.

Method and materials

In current case–control study, the association between EGFR (− 216G/T), Exo1 (K589E) and LEP (− 2548G/A) gene polymorphisms and lung cancer risk were evaluated by using PCR–RFLP technique and bioinformatics approach analysis.

Study subjects

This study designed to assess the association between the *EGFR* (− 216G/T), *Exo1* (K589E) and *LEP* (− 2548G/A) gene polymorphisms with risk of lung cancer in the Iranian population. A total of 100 patients with lung cancer and 100 age and gender-matched healthy controls were recruited for the study from the Ayatollah Khansari Hospital, Arak City, Iran, from January 2019 until April 2020. Lung cancer of patients was diagnosed by an oncologist and was confirmed by histopathology examination. The exclusion criteria of the control group were previous malignancy or genetic diseases. Clinical data were collected from the patient's medical records and demographics information were collected through a questionnaire. The study was approved by the Research Ethics Committees of Baqiyatallah University of Medical Sciences.

PCR–RFLP analysis

DNA extraction of blood samples done with DNG-plus kit (Cinna Gen, Iran) according to the manufacturer's instructions and stored at − 20 °C until molecular analysis. The SNPs of the *EGFR* (rs712829), *Exo1* (rs1047840) and *LEP* (rs7799039) genes were analyzed with PCR–RFLP method. The sequences of the primers used for PCR amplification and PCR conditions for each SNP are shown in Table 1. The PCR products were studied after digestion with *BseRI* (Fermentaz, USA), *Mse I* (Thermo Fisher Scientific, UK) and *HhaI* (Fermentaz, USA) restriction enzymes for − 216G/T of *EGFR* gene (cut from 240 bp G type into 180 + 60 bp T type), K589E of *Exo1* gene (cut from 306 bp G type into 196 + 110 bp A type) and − 2548G/A of *LEP* gene (cut from 281 bp A type into 172 + 109 bp G type), respectively. Digestion reaction conditions were performed per manufacturer recommendations and the fragments obtained from enzymatic digestion were recorded with gel documentation system. Statistical analysis was performed by SPSS version 16. The $P < 0.05$ was considered as the level of significance. The odd ratios (OR) with their corresponding 95% confidence intervals (CI) were calculated by binary logistic regression to study of the associations of SNPs studied with the risk of lung cancer.

In silico analysis

The NCBI SNP database (https://www.ncbi.nlm.nih.gov/ SNP/) was used to access studied SNPs information including the FASTA format of the DNA and protein sequences of the *EGFR* (− 216G/T), *Exo1* (K589E) and *LEP* (− 2548G/A) gene polymorphisms. The non-synonymous SNP (nsSNP) were analyzed using various bioinformatics tools (SIFT, PolyPhen-2, PhD-SNP, SNPs&GO, I-Mutant, MUpro and Project HOPE). The SNPs at the UTR regions were analyzed using FastSNP and SNP Function Prediction tools. The flowchart of the bioinformatics software's used in this study were shown in Fig. 1.

GeneMANIA (http://www.genemania.org/) is online tool software which predicts gene function, physical interaction, genetic interactions, gene co-expression, co-localization, shared protein domains, and pathway involved [40]. Several online-based tools were employed to determine prediction of nsSNPs studied including Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/), Polymorphism Phenotyping-v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph 2/), Predictor of human deleterious single nucleotide polymorphism (PhD-SNP) (http://snps.biofold.org/phd-snp/phd-snp.

Table 1 The primer pairs for each SNP and their characteristics

Gene	SNP	Primer pairs	PCR conditions	Product size (bp)
EGFR	rs712829	F: 5′-GAGCTAGACGTCCGGGCA-3′ 5′-GCTCTCCCGATCAATACTGGA-3′ R:	35 cycles: 94 °C for 10 min, 94 °C for 1 min, 64 °C for 45 s, 72 °C for 45 s, 72 °C for 5 min	240
Exo1	rs1047840	5′-GACACAGATGTAGCACGTAA-3′ F: 5′-CTGCGACACATCAGACATAT-3′ R:	35 cycles: 94 °C for 10 min, 94 °C for 1 min, 56 °C for 45 s, 72 °C for 45 s and 72 °C for 5 min	306
LEP	rs7799039	5′-TAAGCCAAGGCAAAATTGAG-3′ F: 5′-CTTCAAAATTTATGTTCCTCTGC-3′ R:	35 cycles: 94 °C for 10 min, 94 °C for 1 min, 55 °C for 45 s,72 °C for 45 s and 72 °C for 5 min	281

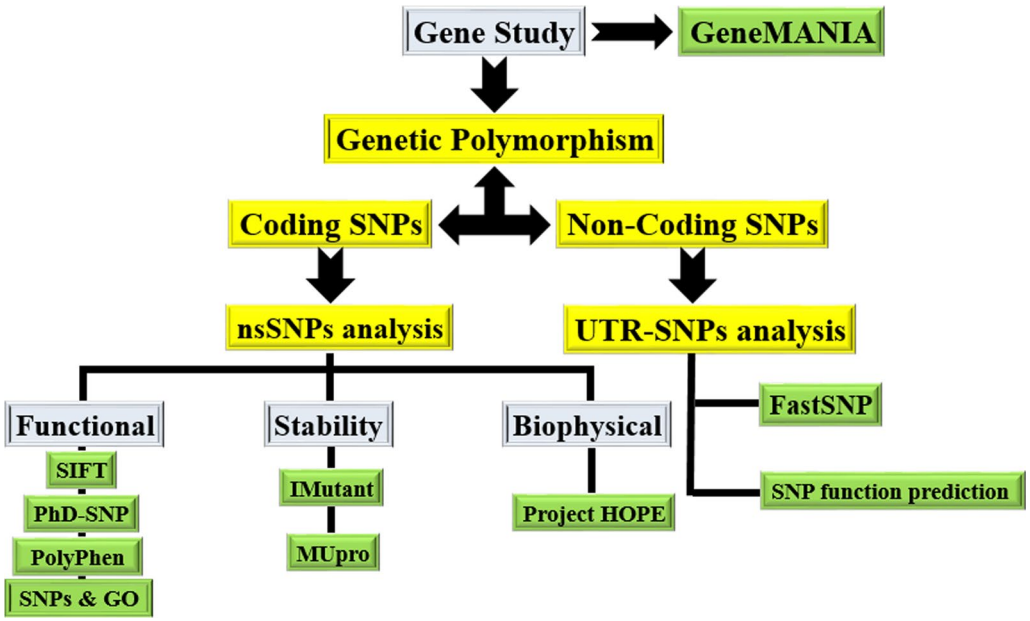

Fig. 1 Diagram of bioinformatics software's used to In Silico analysis process

html), and SNPs&GO (GO-Gene Ontology) (http://snps-and-go.biocomp.unibo.it/snps-andgo/) web-based tools. SIFT gives a probability score. The score less than or equal to the threshold of 0.05 is deleterious, and a prediction greater than the threshold is tolerant [41]. PolyPhen v2 predicts the possible effect of nsSNPs based on sequence, structural conformation. The output of the PolyPhen v2 is a numerical score ranging from 0.0 (benign) to 1.0 (damaging) [42]. PhD-SNP is based on support vector machines (SVMs) which predicts whether a point mutation is a neutral polymorphism or is associated to genetic disorders in humans [43]. SNPs & GO is a method applied to predict whether an amino acid substitution may be related to diseases or not. The prospect score higher than 0.5 detects that the SNP is disease related [44]. For analysis the stability of protein due to the nsSNPs, two online servers were used: I-Mutant 3.0 (http://gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi) and MUpro (http://mupro.proteomics.ics.uci.edu/). I-Mutant 3.0 gives the output as a DDG value which is categorized into one of the three predictions: largely unstable (DDG < −0.5 kcal/mol), largely stable (DDG > 0.5 kcal/mol), or neutral (−0.5 ≤ DDG ≤ 0.5 kcal/mol). MUpro tool calculates a score between − 1 and 1 as the confidence of prediction. The confidence score < 0 indicates that the mutation decreases the protein stability, while a confidence score > 0 means that the mutation increases the protein stability [45, 46]. In order to analysis the biophysical and structural of protein due to the nsSNPs, Project HOPE (https://www3.cmbi.umcn.nl/hope/) were used [47]. For analyze the SNPs located within the UTR region, SNP function prediction SNPinfo (FuncPred) (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm) and FASTSNP (http://FASTSNP.ibms.sinica.edu.tw) were used [48]. The score is given on the basis of levels of risk with a ranking of 0, 1, 2, 3, 4, or 5. This signifies the levels of no, very low, low, medium, high, and very high effect, respectively [49].

Results

PCR–RFLP results

The frequency distributions of selected characteristics of control and lung cancer group with TNM information are shown in Table 2. The age and sex of patients and controls were matched. The mean age of the lung cancer patients and the controls were 62.45 (standard deviation, SD = 11.47) and 61.5 (SD = 10.70) years, respectively. The results of comparing the age range between the two groups shows that, there was no statistical significance difference in the case and control groups ($P = 0.429$). Also, there was no significant statistical difference between male or female among two groups ($P = 0.753$). There was significant statistical difference between the smoking status and the risk of lung cancer among two case and control groups ($P = 0.004$). According to Table 2, the tumor grade was assessed and the majority of the patients were in grade IV. Sixty percent of the patients were positive for lymph node involvement and 70% of patients were metastatic positive.

Table 2 Characteristics of control and lung cancer groups with TNM information

Variable	Defined status	Cases, *n* (%) *N* = 100	Controls, *n* (%) *N* = 100	*P**
Age (year)	> 60	54 (54)	48 (48)	
	≥ 60	46 (46)	52 (52)	0.429
	Mean (SD)	62/45 (11/47)	61/5 (10/70)	
Sex	Male	74 (74)	68 (68)	
	Female	26 (26)	32 (32)	0.753
Smoking status	Yes	42 (42)	21 (21)	
	No	58 (58)	79 (79)	0.004
	Type of LC*	**Tumor grade (T)**	**Lymph node (N)**	**Metastatic status (M)**
Patients, *N* (%)		T1 = 12 (12)	N0 = 40 (40)	
	NSCLC = 50 (50)	T2 = 28 (28)	N1 = 22 (22)	Positive = 70 (70)
	SCLC = 50 (50)	T3 = 25 (25)	N2 = 17 (17)	Negative = 30 (30)
		T4 = 35 (35)	N3 = 21 (21)	

P p-value < 0.05, *LC* Lung Cancer

The PCR–RFLP results of studied SNPs are shown in Fig. 2. The frequency of the alleles and genotypes for the rs712829 (*EGFR*), rs1047840 (*Exo1*) and rs7799039 (*LEP*) between the lung cancer and controls patients is shown in Table 3. For rs712829 of *EGFR* gene, there was a significant statistical difference in TT frequency among lung cancer patients compared to controls ($P = 0.000$, OR = 5.500, CI = 95%, 2.239–13.510). Results of the allele analysis for rs712829 show that there was a significant statistical difference between G allele and risk of lung cancer, in contrast the T allele show a protective role against the risk of lung cancer ($P = 0.001$, OR = 2.976, CI = 95%, 1.557–5.691).

According to Table 3, the frequencies genotypes and allele's analysis for rs1047840 of *Exo1* gene show that the frequency of GG genotype in the case and control groups, was 58% and 78%, respectively, versus the frequency of AA genotype in the cases is 4 times more than controls

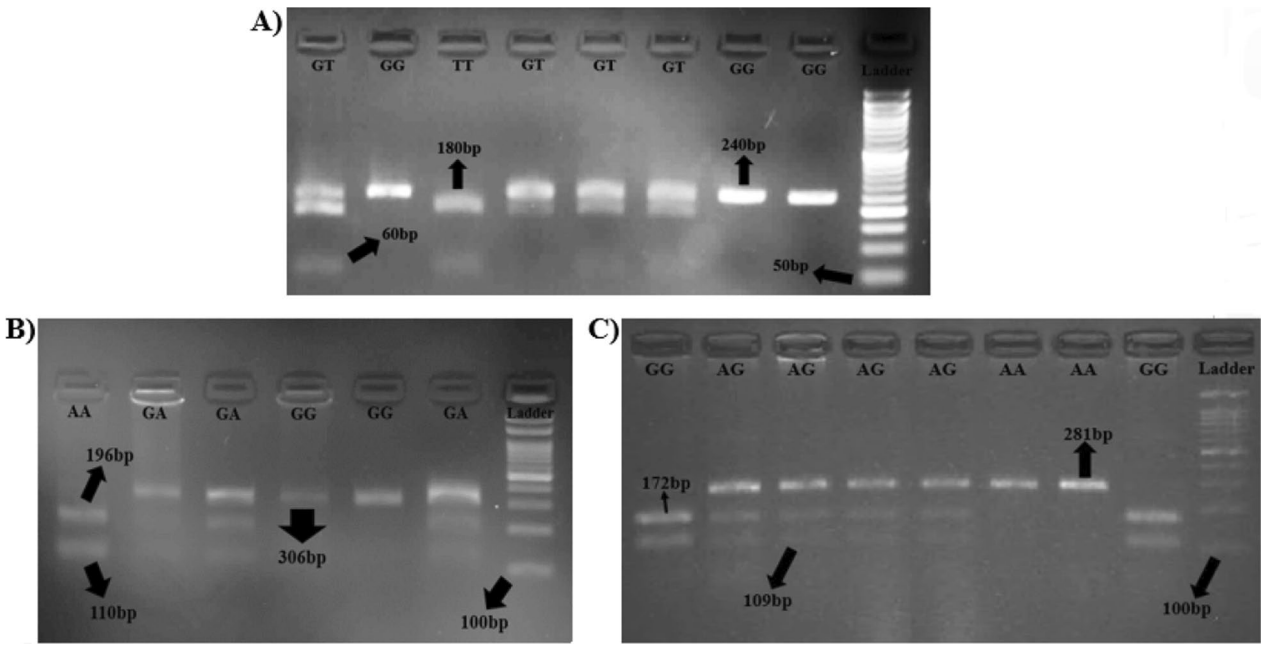

Fig. 2 The PCR–RFLP results of studied SNPs that shown by 3% agarose electrophoresis. **A** Results PCR–RFLP analysis of the *EGFR* rs712829 polymorphism with *BseRI* restriction enzyme at 37 °C/3 h. **B** Results PCR–RFLP analysis of the *Exo1* rs1047840 polymorphism with *MseI* restriction enzyme at 65 °C/3 h. **C** Results PCR–RFLP analysis of the *LEP* rs7799039 polymorphism with *HhaI* restriction enzyme at 37 °C/2 h

Table 3 The frequency of the alleles and genotypes for SNPs studied in cases and the controls

Gene	SNP	Genotype	Cases, n (%)	Controls, n (%)	OR*	95% CI*	*P**
EGFR	rs712829	GG	30 (30)	20 (20)	1.00 (Reference)	1.00	
		GT	55 (55)	25 (25)	0/682	0/299–1/555	0/362
		TT	15 (15)	55 (55)	5/500	2/239–13/510	0/000
		Alleles					
		G	115 (57/5)	62 (31)	1.00 (Reference)	1.00	
		T	85 (42/5)	138 (69)	2/976	1/557–5/691	0.001
Exo1	rs1047840	AA	20 (20)	5 (5)	1.00	1.00	
		GA	22 (22)	17 (17)	3/111	0/848–11/408	0/087
		GG	58 (58)	78 (78)	5/391	1/690–17/200	0/004
		Alleles					
		A	62 (31)	27 (13/5)	1.00 (Reference)	1.00	
		G	138 (69)	173 (86/5)	2/851	1/291–6/300	0.010
LEP	rs7799039	GG	16 (16)	13 (31)	1.00 (Reference)	1.00	
		GA	53 (53)	50 (53)	0/808	0/318–2/050	0/653
		AA	31 (31)	37 (16)	0/641	0/240–1/709	0/374
		Alleles					
		G	75 (37/5)	85 (42/5)	1.00 (Reference)	1.00	
		A	125 (62/5)	115 (57/5)	0/812	0/431–1/530	0.519

P *p*-value < 0.05, *CI* Confidence Interval, *OR* Odds Ratio

group. The frequency of A allele in the case and control groups, was 31% and 13/5%, respectively. The frequencies genotypes and allele's analysis for rs1047840 of *Exo1* gene in cases and the controls show that A allele of this polymorphism associated with risk of lung cancer ($P=0.010$, OR = 2.851, CI = 95%, 1.291–6.300), in contrast the GG genotype and G allele show a protective effect against the risk of lung cancer ($P=0.004$, OR = 5.391, CI = 95%, 1.690–17.200). For rs7799039 of *LEP* gene, no statistically significant difference was observed between the genotypes of this polymorphism in the case and control groups. Also, no statistically significant difference was observed between allele frequencies of the rs7799039 polymorphism in the case and control groups ($P=0.519$, OR = 0.812, CI = 95%; 0.431–1.530).

The result of association between the genotypes of the SNPs studied and TNM staging system of patients are shown in Table 4. There was no significant statistical difference between the genotypes of rs712829 and rs1047840 polymorphism with tumor grade, in contrast the GA genotype of rs7799039 polymorphism was significantly associated with increase tumor grade in the patients ($P=0.024$, OR = 0.222, CI = 95%, 0.060–0.824). According Table 4, the results showed that there was no significant difference between the genotypes of the studied SNPs with lymph node involvement and metastasis in the patients.

In silico analysis results

We investigated rs712829 (*EGFR*), rs1047840 (*EXO1*) and rs7799039 (*LEP*) gene polymorphisms using NCBI dbSNP database. The rs1047840 (K589E) were a coding and non-synonymous SNP that located on exon12 of the *Exo1* gene. The rs712829 (− 216G/T) of *EGFR* gene and rs7799039 (− 2548G/A) of *LEP* gene as non-coding SNPs were in the 5′-UTR and 2KB Upstream, respectively.

GeneMANIA builds a multiplex gene–gene functional interaction network. The interaction network of the *Exo1*, *EGFR* and *LEP* genes predicted by GeneMANIA is shown in Figs. 3, 4 and 5, respectively.

In order prediction of SNPs pathogenicity, the rs1047840 of *Exo1* gene as nsSNP were predicted by various bioinformatics tools. The result prediction was shown in Table 5.

The result protein stability analysis of the rs1047840 of *Exo1* gene, by I-Mutant 3.0 and MUpro servers were shown in Table 6.

The existence of K589E mutant (rs1047840 of *Exo1* gene) and substitution of glutamic acid might harshly disrupt a special conformation of EXO1 protein (Fig. 6).

The results obtained by Project HOPE software for rs1047840 of *Exo1* gene were shown in Table 7.

Table 4 The analysis association between genotypes of SNPs studied with TNM staging system

Gene	SNP	Genotype	Tumor grade		OR*	95% CI*	*P**
			T1 + T2, *n* (%)	T3 + T4, *n* (%)			
EGFR	rs712829	GG	14 (14)	16 (16)	1.00 (Reference)	1.00	
		GT	24 (24)	31 (31)	0.898	0.330–1.442	0.833
		TT	2 (2)	13 (13)	0.236	0.042–1.317	0.100
Exo1	rs1047840	AA	9 (9)	11 (11)	1.00 (Reference)	1.00	
		GA	11 (11)	11 (11)	1.286	0.332–4.972	0.716
		GG	20 (20)	38 (38)	0.686	0.215–1.186	0.524
LEP	rs7799039	GG	10 (10)	6 (6)	1.00 (Reference)	1.00	
		GA	14 (14)	39 (39)	0.222	0.060–0.824	0.024
		AA	16 (16)	15 (15)	0.677	0.153–1.652	0.576
Gene	**SNP**	**Genotype**	**Lymph node status**		**OR***	**95% CI***	***P****
			N−, *n* (%)	N+, *n* (%)			
EGFR	rs712829	GG	12 (12)	18 (18)	1.00 (Reference)	1.00	
		GT	23 (23)	32 (32)	0.969	0.353–1.661	0.952
		TT	5 (5)	10 (10)	0.700	0.164–1.481	0.629
Exo1	rs1047840	AA	10 (10)	10 (10)	1.00 (Reference)	1.00	
		GA	7 (7)	15 (15)	0.500	0.125–1.009	0.327
		GG	23 (23)	35 (35)	0.643	0.205–1.020	0.449
LEP	rs7799039	GG	5 (5)	11 (11)	1.00 (Reference)	1.00	
		GA	20 (20)	33 (33)	0.722	0.191–1.737	0.632
		AA	15 (15)	16 (16)	0.481	0.117–0.982	0.311
Gene	**SNP**	**Genotype**	**Metastatic status**		**OR***	**95% CI***	***P****
			M−, *n* (%)	M+, *n* (%)			
EGFR	rs712829	GG	10 (10)	20 (20)	1.00 (Reference)	1.00	
		GT	17 (18)	38 (38)	0.933	0.323–1.693	0.898
		TT	3 (3)	12 (12)	0.400	0.070–2.277	0.302
Exo1	rs1047840	AA	5 (5)	15 (15)	1.00 (Reference)	1.00	
		GA	10 (10)	12 (12)	0.400	0.555–0.881	0.241
		GG	15 (15)	43 (43)	0.959	0.286–1.920	0.932
LEP	rs7799039	GG	6 (6)	10 (10)	1.00 (Reference)	1.00	
		GA	15 (15)	38 (38)	0.490	0.174–0.954	0.502
		AA	9 (9)	22 (22)	0.422	0.151–0.869	0.512

P p-value < 0.05, *CI* Confidence Interval, *OR* Odds Ratio

Discussion

Among all the cancers, lung cancer with over 1.3 million deaths per year. In Iran, lung cancer is the third most common type of cancer and its prevalence is increasing rapidly [7, 16]. SNPs that lead to protein insufficiency can be associated with an increased risk of cancer development [50]. A large number of genes associated with lung cancer contain SNPs. SNPs are located in gene promoters, exons, introns as well as 5' and 3'-UTRs and affect gene expression, structure and function of protein by various mechanisms. Several studies in different populations have been published suggesting that SNPs of the *EGFR* (− 216G/T), *Exo1* (K589E) and *LEP* (− 2548G/A) genes are associated with lung cancer risk [12–14]. Among the polymorphisms investigated as In vitro analysis, we found that variant genotypes of *EGFR* − 216G/T and *Exo1* K589E were significantly associated with a higher susceptibility of lung cancer, in contrast no significant association was observed between *LEP* (− 2548G/A) polymorphism and the risk of lung cancer. The result of analysis association between genotypes of SNPs studied with TNM staging system were showed that there is a significant association between the GA genotype of rs7799039 polymorphism with increase tumor grade.

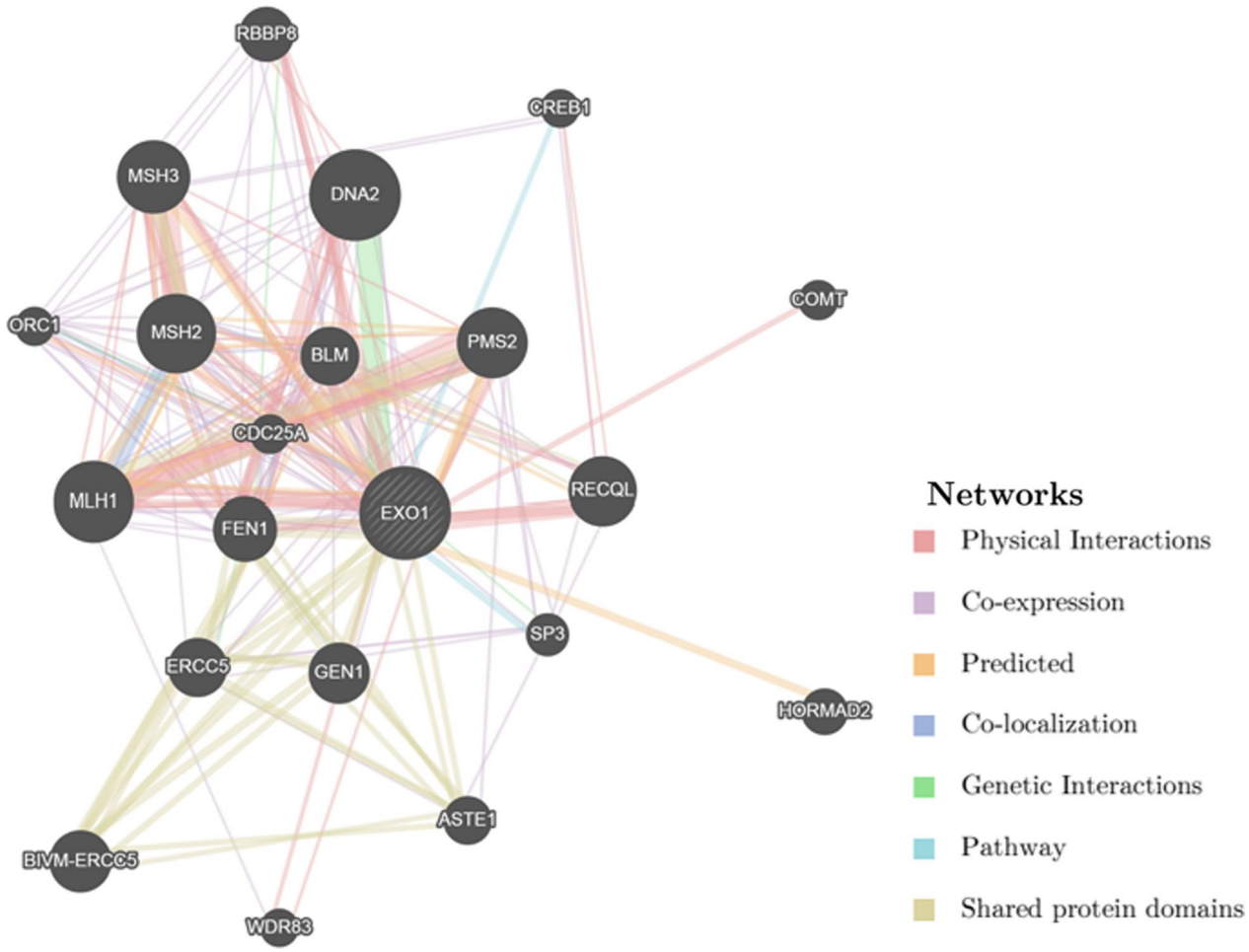

Fig. 3 Gene–gene interaction network of the *Exo1* gene predicted by GeneMANIA

Results of in silico analysis showed that the rs1047840 of *Exo1* gene as nsSNP associated with risk of lung cancer. Also, in silico analysis showed that the rs712829 and rs7799039 may be associated with risk of lung cancer. For the understanding of cellular processes gene–gene functional interaction network is a fundamental factor. GeneMANIA plays an importance role to description of genes co-expression, co-localization, shared protein domains, physical and genetic interactions. In the present study, this database revealed that the existence of rs712829 on *EGFR*, rs1047840 on *Exo1* and rs7799039 on *LEP* gene, can alters the biological processes of these genes and disruption of these pathways, so that may be associated with risk of lung cancer. Among variants of *EGFR* gene rs712829 is a functional polymorphism in the *EGFR* promoter [16, 17]. The results in our study showed a significant association between rs712829 of *EGFR* gene and lung cancer risk. Several studies have demonstrated that the expression and activity of the *EGFR* is highly modified by several polymorphic regions within the *EGFR* gene [12, 16, 49, 51]. The rs712829 polymorphism is located in the promoter region of the *EGFR* gene and was shown in previous studies to modify the expression of the *EGFR* gene and may be associated with risk of lung cancer [12, 51]. According to the results in this study, the frequency of the TT genotype of rs712829 was higher in the control group compared to patients with lung cancer. The presence of the T allele can be protective against lung cancer, so that people who carry two copies of T allele are at a lower risk of susceptibility to lung cancer. In contrast, the presence of the G allele of rs712829 is associated with an increased risk of lung cancer, so that people who carry GG genotype of rs712829 are at a higher risk of susceptibility to lung cancer. This observation is consistent with the results of Nabil et al. [12], who found that the − 216G allele of rs712829 increased lung cancer risk in the Jordanian population. In contrast, a lack of association between the rs712829 of *EGFR* and lung cancer was reported in a United States [49] and Japanese population [51]. Also, in the study conducted by Torres-Jasso et al.

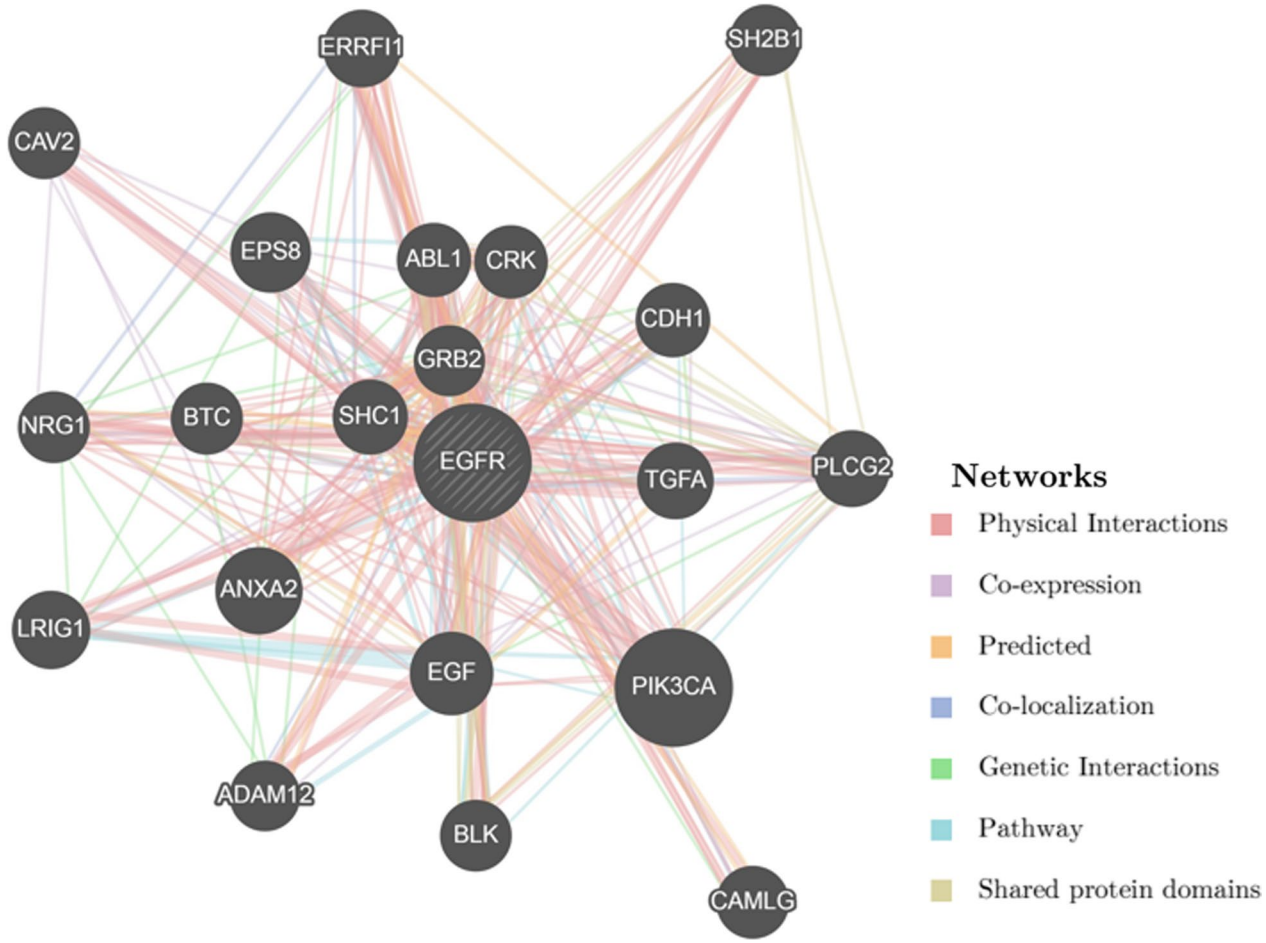

Fig. 4 Gene–gene interaction network of the *EGFR* gene predicted by GeneMANIA

[51] it was found that the TT genotype of rs712829 associated with an increased risk of gastric cancer in a Mexican population. The rs712829 of *EGFR* gene, a functional variant in the *EGFR* promoter, is located in the Sp1 recognition site where several protein factors and transcriptional start sites have been identified. Since the Sp1 binding site is a region that is important for the regulation of *EGFR* transcription, the replacement of G by T at position − 216 increases promoter activity by 30%, thereby resulting in a higher *EGFR* expression level [15]. The rs712829 polymorphism has been shown to influence the response to EGFR-TKIs therapy in Chinese [52] and Korean lung cancer patients [53]. Result of in silico analysis in this study indicated that the rs712829 is a non-coding SNP which were in the 5′-UTR of *EGFR* gene. In silico analysis performed by FastSNP and SNP Function Prediction showed that the rs712829 can alter promoter regulation by effect on transcription factor-binding site and alter splicing by effect on exonic splicing enhancer or exonic splicing silencer of *EGFR* gene and may be associated with the risk of lung cancer [48]. The rs712829 can have important effect on mRNA processing, transcription activity, stability, and protein translation, therefore, it can play a fundamental role in the development of lung cancer [54]. Among SNPs of *Exo1* gene, the rs1047840 is most important variant associated with the risk of various cancers [16, 19, 21–23]. The rs1047840 of *Exo1* is located on exon 12, and its variation leads to a change in the 589th amino acid of the *Exo1* protein from lysine to glutamic acid, which might affect *Exo1* expression (52). Some studies show that the A allele of the rs1047840 polymorphism may influence *Exo1* activity and may be associated with risk of cancer. For a person who has a risk-imparting genetic variant, such as the A allele of the rs1047840 polymorphism, that variant will likely synergistically increase their cancer risk [24, 55]. For the rs1047840 of *Exo1* gene, we found that A allele were significantly associated with increased risk of lung cancer, versus individuals who carry GG genotype are at a lower risk of susceptibility to lung cancer, so that G

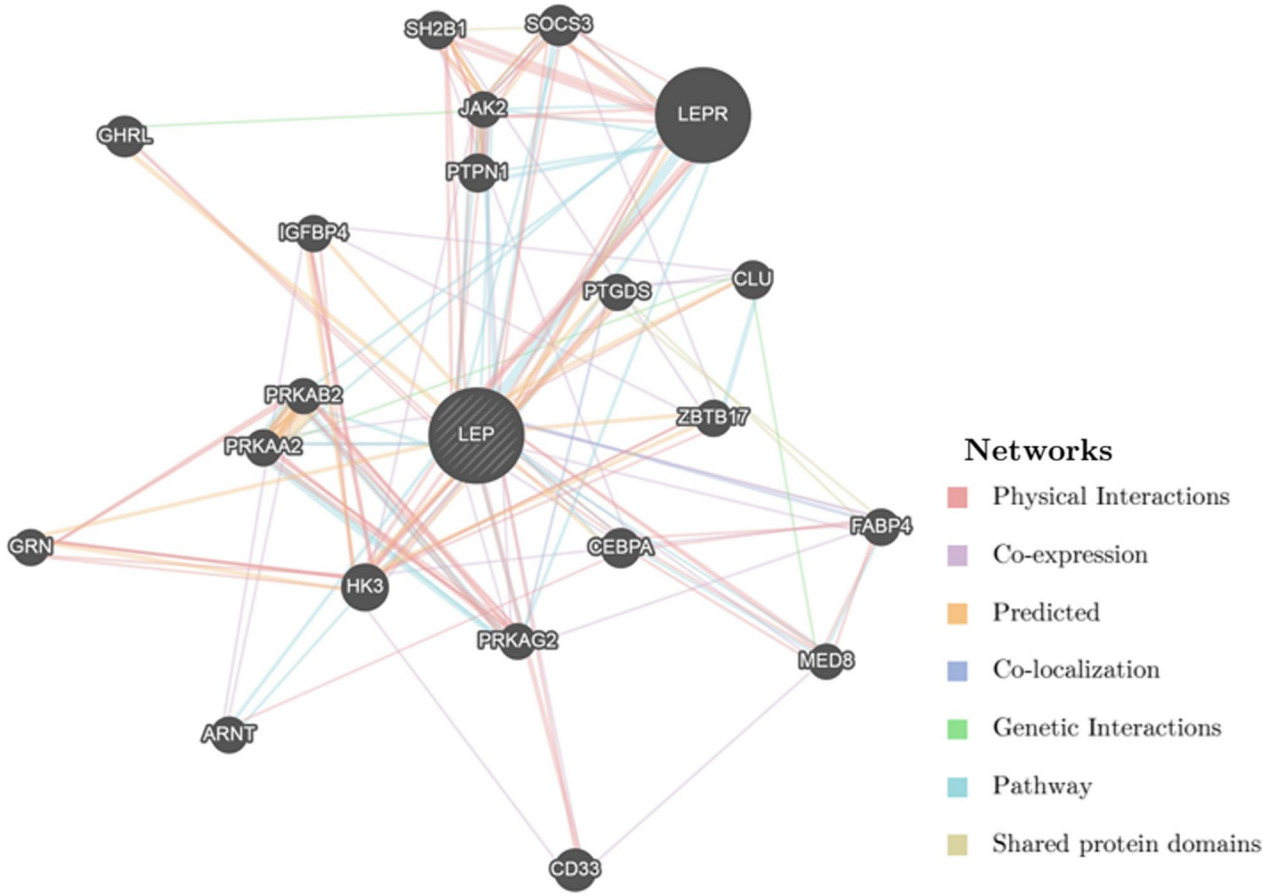

Fig. 5 Gene–gene interaction network of the *LEP* gene predicted by GeneMANIA

Table 5 Prediction of rs1047840 of *Exo1* by various bioinformatics tools

Gene	SNP ID	Allele change	Amino acid change	SIFT prediction (score)	PolyPhen-2 prediction (score)	PhD-SNP prediction (score)	SNPs & GO prediction (score)
EXO1	rs1047840	A→G	K589E	Tolerated (0.585)	Benign (0)	Neutral (0.476)	Disease (0.727)

Table 6 Analysis of protein stability the rs1047840 of *Exo1* gene by MUpro

Gene	SNP ID	Amino acid change	I-Mutant 3.0			MUpro		
			DDG Value	Stability	RI	DDG Value	Stability	CS
EXO1	rs1047840	E589K	− 0.71	Decrease	5	− 1.24842	Decrease	− 0.407

RI Reliability Index, *CS* Confidence Score

allele of this polymorphism has a protective role against lung cancer. The finding in our study about rs1047840 of *Exo1* gene is consistent with the results of Jin et al. [13] in the Chinese population. Also, in the meta-analysis study conducted by Tang et al. [20] was found that the A allele compared to G allele, was associated with 1.18 times increased risk of lung cancer, so that the A allele of *Exo1* rs1047840 may confer modulating effects on the risk of lung cancer and can be used as a marker for early detection. In the study conducted in Iranian population by Nasserinejad et al. [56] was found that the A allele associated with increased risk of colorectal cancer that is

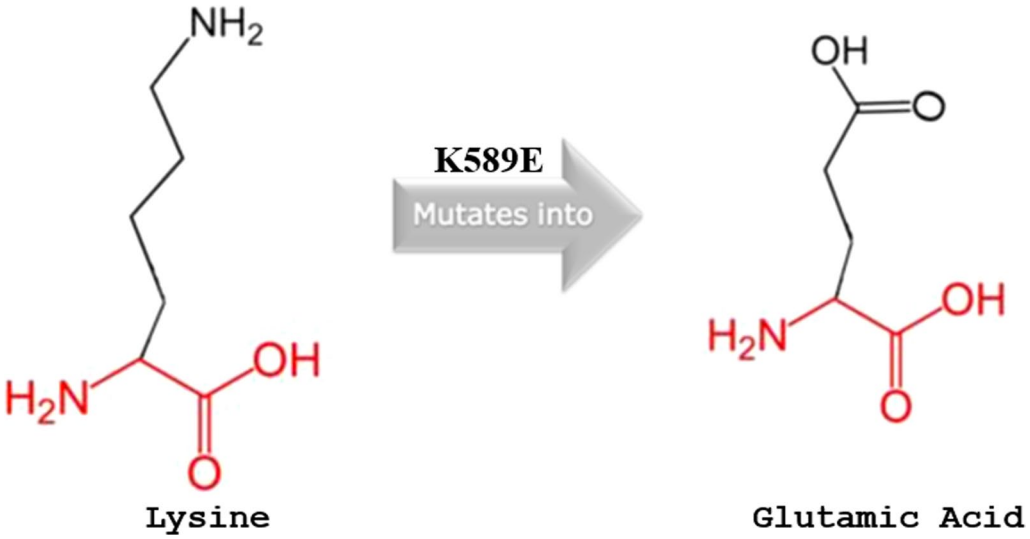

Fig. 6 Schematic structures of the original (left) and the mutant amino acid of K589E by HOPE software

Table 7 Interpretation of the impact of rs1047840 on Exo1 protein structure and stability by HOPE

Gene	SNP ID (Amino Acid Change)	Size	Charge	Hydrophobicity	Interpretation
EXO1	rs1047840 (E589K)	W > M	Positive > Negative	Decrease	The mutated residue is located in a domain that is important for the main activity of the protein. This mutation might lead to loss of function and interactions

The results obtained by SNP Function Prediction and FastSNP software's were shown in Tables 8 and 9, respectively

Table 8 Analysis of rs712829 and rs7799039 by SNP Function Prediction

dbSNP ID	Chromosome (Gene)	Position	Allele	TFBS	Splicing(site)	Splicing (ESE or ESS)	Conservation
rs712829	7 (EGFR)	55,054,249	G/T	Yes	–	Yes	0.001
rs7799039	7 (LEP)	127,666,019	A/G	Yes	–	–	0.000

TFBS: Transcription factor-binding site, ESE: Exonic splicing enhancer, ESS: Exonic splicing silencer

Table 9 Analysis prediction functionally significant rs712829 and rs7799039 by FastSNP

Gene	SNP ID	Nucleotide change	Position	Level of risk	Possible functional effect
EGFR	rs712829	G > T	5′ UTR	Very Low–Low (1–2)	Effect on splicing and promoter regulation
LEP	rs7799039	G > A	2KB Upstream	Very low–medium (1–3)	Effect of promoter regulation

consistent with result in our study. Luo et al. [52] identified that the A allele of the *Exo1* rs1047840 polymorphism was significantly related to an increased cervical cancer risk compared with the G allele. Duan et al. [57] show that the A allele of rs1047840 polymorphism may be applied as a novel biomarker for tumor susceptibility. Similarly, many other studies are consistent with this association of the *Exo1* rs1047840 with breast cancer, oral cancer and gastric cancer [21–23]. However, Zienolddiny et al. [25] have shown no significant association

of the rs1047840 polymorphism and non-small cell lung cancer risk. The inconsistent results related to the association between rs1047840 and lung cancer may be due to different ethnics and explained by the differences in the studied populations. Also, for accurate analysis association of thispolymorphism with lung cancer in Iranian population, other information associated with lung carcinogenesis like smoking exposures, family history of cancer in first-degree relatives, occupational exposures and certain dietary factors should be considered. Result of in silico analysis about rs1047840 showed that this SNP is a coding and non-synonymous SNP that located on exon12 of the *Exo1* gene. A non-synonymous SNP that changes amino acid sequence of the protein–protein interface can alter protein interactions, affect stability and alter post-translational modifications [58]. The rs1047840 as a non-synonymous SNP result in amino acid substitution (K589E) that can affect *Exo1* protein structure and function. The prediction of rs1047840 pathogenicity analysis by SNPs & GO prediction indicated that this SNP associated with the incidence of lung cancer. Protein stability is necessary for the structural and functional activity of a protein [59]. According result of I-Mutant 3.0 server, nsSNP rs1047840 of *Exo1* gene showed a DDG value of less than −0.5, which indicated that they were largely unstable and decreased protein stability. Also, results obtained with MUpro software showed that the existence of rs1047840 in *Exo1* gene causes decrease protein stability of the Exo1 protein structure. Protein stability governs the conformational structure of the protein and thus determines the function. Any change in protein stability may cause degradation or aberrant conglomeration of proteins. In other words, the function, activity and regulation of a protein significantly depend on the structural stability of the molecule, therefore decrease in protein stability causes misfolding and aggregation of proteins leading to dysfunction [60]. Result of protein stability about rs1047840 indicated that the existence of rs1047840 in *Exo1* gene causes decrease protein stability of the Exo1 protein structure therefore, it can increase the risk of tumorgenesis. Biophysical and structural analysis of nsSNP rs1047840 by Project HOPE software indicated the mutated residue is located in a domain that is important for the main activity of the protein. Mutation of the residue might disturb this function. The mutation introduces a residue with a charge opposite to the wild-type. This can cause repulsion with other residues in the protein or ligands. Also, this mutation might lead to loss of interactions. In other words, the existence of rs1047840 (K589E) in *Exo1* gene influence lung cancer susceptibility due to changes in the structure, function and interactions of the Exo1 proteins. A role for leptin in lung cancer etiology is proposed by observations from the different studies, in which an increased risk of lung cancer was associated with the overexpressing genetic variant in *LEP* gene [14]. Several studies indicated that *LEP* rs7799039 are correlated with the development of cancers. The existence of rs7799039 polymorphism in *LEP* gene associated with high gene expression and twofold LEP secretion. Higher mRNA expression of *LEP* gene may increase risk of lung cancer [14, 29–33]. In our study there was no significant association between rs7799039 polymorphism of *LEP* gene and the risk of lung cancer that is consistent with the results of meta-analysis by Juan et al. [16] about colorectal cancer [16]. Also, in the study conducted by Hung et al. [33] it was found that there is no association between rs7799039 polymorphism of *LEP* gene and the risk of oral cancer in Taiwan population. However, this observation in our study is inconsistent with the results of Ribeiro et al. [14], who found that the rs7799039 polymorphism increased lung cancer risk in the Portugal population. Marcello et al. [60] identified that the A allele of the rs7799039 polymorphism was significantly related to an increased thyroid cancer risk in Brazilian population. Moreover, Ribeiro et al. [14] identified that the A allele of the rs7799039 polymorphism was significantly associated to an increase prostate cancer risk. The significant association of rs7799039 polymorphism with the risk of breast cancer has been reported by Mohammadzadeh et al. [50] in the Iranian female population. Our results suggest that individuals with lung cancer that carry GA genotype of rs7799039 are associated with increase grade tumor. This SNP is located in the promoter region, this polymorphism may affect gene expression at a transcriptional level, leading to more or less leptin production [60]. Probably the high expression of leptin gene in patients with GA genotype is associated with an increase in tumor grade. These findings suggest that leptin may have an important role in tumor cells growth and inducing a carcinogenic environment. In Silico analysis indicated that the rs7799039 is a non-coding SNP which were in the 2KB Upstream promoter of *LEP* gene. Promoter SNPs located in transcription factor binding sites are associated with gene expression changes and regional histone modifications [61]. FastSNP and SNP Function Prediction analysis showed that the rs7799039 can alter promoter regulation by effect on transcription factor-binding site of LEP gene and may be associated with the risk of lung cancer. The rs7799039 in promoter of *LEP* gene can increase transcription activity (high expression mRNA), therefore, it may be associated with the development of lung cancer. Further studies on this topic are required in order to clarify rs7799039 of *LEP* gene and lung cancer etiopathogenesis. The inconsistent finding about SNPs investigated in our study in the Iranian

population compared to various studies in other populations can be explained by the differences in the genetic background of the studied populations. Other polymorphisms in the region of the *EGFR*, *ExoI* and *LEP* genes in the studied populations that regulate the effect of the SNPs studied. Analysis of the expression of the *EGFR*, *ExoI* and *LEP* genes in the presence of rs712829, rs1047840 and rs7799039 polymorphisms respectively can give more complete results about association of these SNPs with risk of lung cancer.

Conclusion

In conclusion, translational research that discovered polymorphisms with risk of lung cancer previously established in clinical practice. The rs712829 of *EGFR* and rs1047840 of *Exo1* genes associated with lung cancer among Iranian population can be considered as a risk factor of non-small cell lung cancer for early detection and primary prevention.

Abbreviations

LC	Lung cancer
NSCLC	Non-small cell lung cancer
SCLC	Small cell lung cancer
SNPs	Single nucleotide polymorphisms
EGFR	Epidermal growth factor receptor
Exo1	Exonuclease 1
LEP	Leptin
PCR–RFLP	Polymerase chain reaction fragment length polymorphism
MMR	DNA mismatch repair
TKIs	Tyrosine kinase inhibitors
CI	Confidence interval
OR	Odds ratio
SIFT	Sorting intolerant from tolerant
PHD-SNP	Predictor of human deleterious single nucleotide polymorphism
SNPs&GO	GO-gene ontology
SVMs	Support vector machines
DDG	Delta Delta G
HOPE	Have (y) our protein explained
TFBS	Transcription factor binding sites
ESE	Exonic splicing enhancers
ESS	Exonic splicing silencers
nsSNP	Non-synonymous single nucleotide polymorphism
RI	Reliability Index
CS	Confidence Score

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Author contributions
MP and SMH designed the experiments; MP, AA and JA performed experiments and collected data; MP and SMH discussed the results and strategy; SHM Supervised, directed and managed the study; MP, SMH, AA, and JA Final approved of the version to be published.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved by the Research Ethics Committees of Baqiyatallah University of Medical Sciences, Tehran, Iran (Approval ID: IR.BMSU.REC.1399.312). Informed consent was obtained from all individual participants included in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests regarding the publication of this paper.

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