p21 Gene Expression Evaluation in Esophageal Cancer Patients

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Key Words
Esophageal cancer · Gene expression · Paraffin-embedded tissue · p21 · RT-qPCR

Abstract
Background: Cancer is the third most common cause of death in Iran, and 30,000 Iranians lose their lives each year due to cancer. Esophageal cancer is the eighth most common cancer and the sixth leading cause of death from cancer worldwide. It is reported that more than 80% of all deaths from esophageal cancer occur in developing countries. The p21 protein is encoded by WAF1/CIP1, a tumor suppressor gene located on chromosome 2. p21 is a protein known to be related to the cell cycle; p21 stimulation is a common mechanism of growth inhibition in different physiological conditions.

Summary: The results indicated no significant difference in p21 gene expression between patients and controls (p > 0.05). Moreover, no significant difference was observed in p21 gene expression between males and females (p > 0.05).

Key Message: It appears that p21 is not a specific tumor marker in Iranian esophageal cancer patients.

Practical Implications: In this qualitative research work, for the first time, we investigated the p21 gene expression in esophageal cancer patients in Iran. For the present study, we randomly selected 15 paraffin-embedded esophageal cancer tissues and 15 paraffin-embedded normal esophageal samples collected from different medical centers in Zahedan and Kashan. RT-qPCR reactions were performed with three repetitions for the p21 gene and internal control (GAPDH) using the 2^-ΔΔCt (Livak) method for all samples. Results were analyzed using SPSS software.

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Introduction

Cancer is the second most common cause of death in the United States and the third most common in Iran; 30,000 Iranians lose their lives to cancer each year [1]. Esophageal cancer is the eighth most common cancer and the sixth leading cause of death from cancer worldwide. It is reported that more than 80% of all deaths from esophageal cancer occur in developing countries. Squamous cell carcinoma (SCC) is responsible for more than 90% of esophageal cancer cases in the United States. Esophageal cancer incidence varies significantly according to geography and race, which can be due to differences in exposure to risk factors [2]. SCC accounts for 90% of esophageal cancers in countries on the esophageal cancer band (from East Turkey across northern Iran and the Central Asian republics to northern and central China) [3, 4]. Iranian studies indicate a high risk of esophageal cancer in the coastal areas of the Caspian Sea (northern Iran, Golestan) [5], while the risk of esophageal SCC (ESCC) is more or less constant [6].

The p21 protein is encoded by WAF1/CIP1 [part 1 of wild-type p53/protein 1 reactive to cyclin-dependent kinase (CDK)], a tumor suppressor gene located on chromosome 2.6 p21 [7]. This protein is known to be related to the cell cycle; p21 stimulation is a common mechanism of growth inhibition in different physiological conditions. p21 is observed in aging courses, or in genes involved in diseases associated with aging (Alzheimer’s disease, amyloidosis); reversible and irreversible forms of growth inhibition due to damage and final differentiation to postmitotic cells are stimulated shortly and transiently [8–10]. p21 primarily prevents the progression of the cell cycle by preventing CDK2 activity, which is required for the phosphorylation of retinoblastoma with sequential release and gene activity associated with E2F, the burning of duplicated sources, and the activity of proteins involved in the synthesis of DNA [11]. This activity is also shared with other inhibitors such as P27 and P57; genetic and biochemical evidence suggests that CDK2 plays certain roles in the appearance of tumors [12]. However, p21 is exclusively placed for acting as an inhibitor, which is active in response to cell tissue varieties and environmental signals to develop tumor suppressor activity. Experimental evidence suggests that the proliferation of human cancer cells does not require CDK2 activity [13]. Various immunohistochemical studies have investigated p21 in different tumors. Some studies have shown a relationship between p21 expression and worse prognosis in breast cancer [14], but it has been proven to be less likely to cause worse prognoses in anal [15], bladder [16], and ovarian [17] cancers. The association between the expression of p53, p21, MDM2, and P16 proteins and p21 gene polymorphisms and gene methylation of P16 in esophageal tumor tissue was assessed in patients living in northeast Golestan province. Results revealed an association between smoking in people with cancer and p53 protein expression and polymorphism of the p21 gene [18]. A similar study of the relationship between p53 and p21 and smoking associated the overexpression of p21 with poor prognosis by immunohistochemistry [19]. A study of p21 polymorphism in esophageal cancer and its relationship to smoking showed that two polymorphisms of p21, alone or mixed, are not biomarkers of a genetic predisposition to ESCC [18]. Thus, research findings suggestive of an interaction between environmental factors such as smoking and genetics and molecular mechanisms like methylation of the p16 gene show the polymorphism of the p21 gene and increased p53 protein expression.

Materials and Methods

Paraffin-embedded tissue was sectioned by a microtome device, and slices were placed in 1.5-mm sterile RNase-free tubes. After removing the paraffin, RNA was extracted from the paraffinic tissue by the RNeasy® FFPE Kit (QIAGEN Company) (table 1).

After removing the paraffin, RNA was extracted as described below [20]. First, 240 μl proteinase K digestion buffer was added to the tube and vortexed for 1 min at a speed of 10,000 rpm. Then, 10 ml proteinase
K was added and mixed gently by pipetting. The mixture was incubated for 15 min at 56°C and then placed at 80°C for 15 min. In the next stage, the mixture was centrifuged lightly, and the colorless lower phase was transferred to a new 1.5-mm tube. Tubes containing the samples were incubated on ice for 3 min and then centrifuged for 15 min at a speed of 135,000 rpm. In the next stage, the supernatant solution was transferred into a new 2-ml tube without damaging the deposit. No precipitation was transferred. Then, all DNase Booster Buffer samples (about 25 μl) and 10 μl DNaseI were added based on a 1/10 ratio. The mixture was mixed well by inverting the tubes and centrifuging at low speed for a short time (spinning), so that the surrounding fluid was placed at the end of the tube, and then it was incubated at room temperature for 15 min. Next, 500 μl RBC buffer was added and thoroughly mixed followed by the addition of 1,200 μl 96–100% ethanol. The mixture was mixed well by pipetting and then immediately entered into the next phase of washing. Subsequently, 700 μl of the sample was transferred to RNeasy MinElute® Spin Columns placed in a 2-ml tube; the door was slowly shut, and the sample was centrifuged for 15 s at a speed of 12,000 rpm. The solution inside the tubes was discarded, and the tubes were used again at a later stage. The previous stage was repeated until all samples had passed through the RNeasy MinElute Spin Columns. Then, 500 μl buffer was added to the RNeasy MinElute Spin Columns, the doors were slowly closed, and the buffer was centrifuged for 15 s at a speed of 12,000 rpm. The solution inside the tube was discarded, and the tubes were used again at a later stage. Next, 500 μl buffer was added to the RNeasy MinElute Spin Columns, the doors were slowly closed, and the buffer was centrifuged for 2 min at a speed of 10,000 rpm so that the RNeasy MinElute Spin Columns could be washed (the tubes and the solution inside the tubes were discarded). After centrifugation, the RNeasy MinElute Spin Columns were removed from the tube so that they did not touch the inferior solution and were placed in new tubes; the RNeasy MinElute Spin Column doors were left open, and the solution was centrifuged at maximum speed for 5 min (the tubes and solution inside the tubes were discarded). Finally, RNeasy MinElute Spin Columns were placed on the walls of new 1.5-mm tubes, 30 μl RNase-free water was added directly to the walls, and the doors were gently closed. The solution was incubated for 10 min at room temperature and then centrifuged for 1 min at maximum speed until RNA was extracted. To ensure the quality and quantity of the extracted RNA, concentration and OD were measured by optical spectroscopy, and samples were observed on 2% agarose gel. In the second stage after RNA extraction, DNA was synthesized from it for the next stages. This reaction was performed using the First Strand cDNA Synthesis Kit (K1621; Fermentas Thermo Scientific) (table 2) as detailed below.

Before beginning the tests, all materials in the kit were mixed and centrifuged. Then, all ingredients were placed on ice. 0.1–5 μl of RNA was extracted and increased to 12 μl by adding sterile distilled water (water, nuclease free). In the next stage, tubes were placed at 65°C for 5 min and then placed on dry ice; 4 μl of reaction buffer was added, and then 1 μl of reagent Ribolock RNase Inhibitor (20 U/μl) was added. Two microliters of 10 mM dNTP and 1 μl of RevertAid M-MuLV RT (200 U/μl) were added. The volume inside the tube, 20 μl, was mixed, centrifuged, and then placed at 42°C for 60 min. Then, samples were placed at 70°C

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**Table 1. List of RNA extraction kit components**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer PKD</td>
<td>45 ml</td>
</tr>
<tr>
<td>Buffer RBC</td>
<td>15 ml</td>
</tr>
<tr>
<td>Buffer RPE</td>
<td>11 ml</td>
</tr>
<tr>
<td>K proteinase</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>RNase 0-free Dnase I</td>
<td>1,500 U</td>
</tr>
<tr>
<td>RNase 0-free water</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>DNase Booster Buffer</td>
<td>2 ml</td>
</tr>
<tr>
<td>RNeasy MinElute spin columns (each in a 2-ml collection tube)</td>
<td>50 parts</td>
</tr>
</tbody>
</table>

**Table 2. List of cDNA kit components**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA kit components</td>
<td>20 reactions</td>
</tr>
<tr>
<td>Primer</td>
<td>25 μl</td>
</tr>
<tr>
<td>Water, nuclease-free</td>
<td>2 × 1.25 ml</td>
</tr>
<tr>
<td>5× reaction buffer</td>
<td>150 μl</td>
</tr>
<tr>
<td>Ribolock RNase Inhibitor (20 U/μl)</td>
<td>25 μl</td>
</tr>
<tr>
<td>10 mM dNTP Mix</td>
<td>50 μl</td>
</tr>
<tr>
<td>RevertAid M-MuLV RT (200 U/μl)</td>
<td>25 μl</td>
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</tbody>
</table>
for 5 min and finally kept at \(-20\, ^\circ\)C. Then, cDNA, obtained to quantify gene expression, was assessed by RT-qPCR. For this reaction, the mRNA sequences of the p21 gene were designed according to the primer sequence.

### Stages of Real-Time PCR Reaction

The characteristics of the p21 gene sequence are as follows: locus: NM_000389; linear: PRI 25-MAY-2014 *Homo sapiens* CDK inhibitor 1A (p21, Cip1; CDKN1A), transcript variant 1, mRNA (table 3).

To determine the optimal amount of cDNA, concentrations were prepared at 100, 200, 300, 400, and 500 ng. In this test, the Cinagen HotTaq Evagreen qPCR was used. After determining the optimum amount of cDNA, the amounts of mixture and components for real-time PCR reaction for \(p21\) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (table 4) were prepared as described below. Real-time qPCR reaction was performed to compare \(p21\) gene expression. GAPDH was used as an internal control. The temperature of 54 \(\degree\)C was selected, and the results were evaluated. For the standard curve, several serial dilutions of one sample were prepared for both primers and the reaction by QIAGENE USA Rotorgen, and then the standard curve was drawn by the software, and the reaction output was calculated. To ensure accuracy in sample size, samples were placed on agarose gel. Statistical analysis was performed by the t test and SPSS16 software.

### Results

Fifteen patients with esophageal cancer and 15 healthy subjects were enrolled in the study. Patient samples were taken and matched with the control group regarding gender. Nine participants were male and 6 were female. The control group comprised 15 participants, 9 of whom were men and 6 were women (fig. 1).

### RNA Extraction

The quality of the RNA samples on agarose gel was examined; since RNA was extracted from paraffin-embedded tissue and did not have a high quality, the RNA band can be seen as a smear on the gel (fig. 2). After RNA extraction, the absorption ratio of light at a wavelength of 260–280 nm was used to determine purity, and the absorption ratio at 260 nm was used to determine the concentration. The absorption ratio was calculated to be 1.8–2 for all samples.

### PCR Gradient

To determine the optimum temperature for binding primers, the \(p21\) and GAPDH reference genes were multiplied by the primers and then observed on 2% agarose gel (fig. 2). The optimum temperature was calculated to be 54 \(\degree\)C for the \(p21\) gene (fig. 3) and 58 \(\degree\)C for the GAPDH gene (fig. 4).

<table>
<thead>
<tr>
<th>Table 3. Characteristics of the P21 gene primers</th>
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<tbody>
<tr>
<td><strong>Forward primer</strong></td>
</tr>
<tr>
<td>GACCAGCATGACAGATTTC</td>
</tr>
<tr>
<td>GC%=47.37</td>
</tr>
<tr>
<td>TM: 53.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 4. Characteristics of the reference gene GAPDH primer</th>
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</thead>
<tbody>
<tr>
<td><strong>Reverse primer</strong></td>
</tr>
<tr>
<td>CATGTAGTTGAGGTCAATGAAGG</td>
</tr>
<tr>
<td>TM: 58</td>
</tr>
</tbody>
</table>

*For 5 min and finally kept at \(-20\, ^\circ\)C. Then, cDNA, obtained to quantify gene expression, was assessed by RT-qPCR. For this reaction, the mRNA sequences of the p21 gene were designed according to the primer sequence.*
Fig. 1. Percentage of men and women in the study groups.

Fig. 2. Results of RNA extraction from patient material on 2% agarose gel.

Fig. 3. Determining the optimum temperature for p21 (146 bp).

Fig. 4. Temperature gradient of the GAPDH gene.
Real-Time PCR

Optimal cDNA was observed for real-time PCR reaction. After the reaction, the Ct values of samples were determined for all prepared dilutions by GAPDH primers from the reproduction charts, and then the curves were drawn (based on Ct on the y-axis and log of copies on the x-axis). When the standard curve was drawn, the reaction output was calculated at 0.92 for the GAPDH gene, and the R² degree at 0.96 (fig. 5).

Standard Curve of the p21 Gene

The Ct values of samples were determined for all prepared dilutions by GAPDH primers from the reproduction charts, and the curves were drawn (based on Ct on the y-axis and log
of copies on the x-axis). The standard curve was drawn (fig. 6), and the reaction output was calculated at 0.90 for the p21 gene, and the R² degree at 0.96.

Figure 7 shows the progress curves of the GAPDH gene amplification reaction during each cycle. Here, the number of cycles is indicated on the horizontal axis and the intensity of the fluorescent light emitted from the device on the vertical axis. Figure 8 shows the progress curves of the p21 gene amplification reaction during each cycle. Here, the number of cycles is
Melting Curve Analysis

The main advantage of real-time PCR is the melting curve, with which a variety of products in the PCR process can be determined. To draw the curve, the device changes the temperature of samples at specified intervals to a certain degree. The speed of changes is shown on the y-axis, and the device’s temperature is shown on the x-axis.

Melting Changes the Curve Depending on the Temperature of the GAPDH Gene

To plot the curve, the increase in sample temperature and the light emitted from the samples are measured. In figure 9, a sudden drop in the intensity of fluorescent light can be indicated on the horizontal axis and the intensity of the fluorescent light emitted from the device on the vertical axis.

**Fig. 9.** Fluorescence changes the curve according to GAPDH gene temperature.

**Fig. 10.** Melting curve of the GAPDH gene.
seen at a temperature of 75 °C for the GAPDH gene. In figure 10, each peak represents a product. The melting peak also includes a peak at 76.5 °C and the temperature of the GAPDH gene product.

The sample temperatures increase gradually, and the light emitted from the samples is measured. With the rise in temperature, DNA becomes double stranded, and colored molecules are separated from this product. The intensity of the fluorescent light decreases with temperature increase until the temperature reaches 75 °C, which is the melting temperature of the PCR reaction product. The intensity of the fluorescent light is reduced (fig. 11), and a melting curve is created (fig. 12).
Confirmation of cDNA Synthesis

To ensure proper synthesis, GAPDH was performed for all samples by the p21 gene. In both cases, the existence of a band with the desired size and the absence of nonspecific bands indicated the success of the cDNA synthesis (fig. 13).

After ΔCt was calculated for the p21 and GAPDH genes, the data were analyzed using SPSS software and the t test method. No significant difference was observed in p21 gene expression between patients and controls (p > 0.05) (fig. 14). Moreover, no significant difference was observed in p21 gene expression between males and females (p > 0.05). The analysis of data showed a p value > 0.05, so no statistically significant difference exists in the amount of p21 gene expressed between healthy and sick people in the study groups. In addition to the statistical analysis performed by the t test, ANOVA was used to confirm the results of the test. The results showed no statistically significant difference at the significance level of p > 0.05.

Discussion

Protein p21 exists in low amounts in most cells being reproduced. As protein p53 is activated by DNA fractures or intermediate replicators, it is connected to motifs in promoter CDKN1A (the gene coding the p21 protein) and increases the transcription of the CDKN1A
gene sharply. However, the transcription of the CDKN1A gene is impaired in cancer cells produced during p53 mutations and thus fails to limit the progression of the cell cycle in response to DNA damage and the incomplete cloned chromosome. The intracellular levels of this protein increase in cancerous tissue due to p53 gene mutations and the increased half-life of the mutant form of the protein, but the protein cannot express the p21 gene due to lack of normal function. Regarding the activation pathways of p21 (dependent on p53 and independent of p53), increased expression of p53, cyclin D, Myc, and Ras causes a lack of or reduction in p21 expression. The biological and network adjustment activity of the p21 complex makes predictions about its function to make cancer treatment possible. No relationship between the expression of p21WAF1 and clinical pathological parameters was found regarding sex and age [21].

The cell cycle is crucial for cell survival. Various factors and proteins coordinate, regulate, and control this cycle at many set points in positive or negative ways. In fact, various genes exist in the cells which encode the required proteins for cell cycle control. Extensive studies show a sophisticated correlation between cell cycle time and cancer. Researchers have identified several inhibitor proteins and their function during the cell cycle. Inhibitor proteins ensure the correct DNA replication and cell cycle in normal conditions; obviously, mutations in the genes replicating them or regulating their expression or activity have a central role in the development of cancer. It is generally accepted that multistage genetic and epigenetic changes cause normal cells to mutate into malignant cells. This causes the cells to act independently of the proliferative regulation and cell death cycle and also stimulates the proliferative, infiltrative, and metastatic capacity of cells [22].

One of the main reasons for changes in cells in mammals is the loss of the appropriate cell cycle control. The p21 gene, a CDK inhibitor (also known as p21WAF1/Cip1), stimulates the cell cycle arrest in response to many stimuli [23]. The p21 gene, an important regulator of the cell cycle, binds to PCNA and acts as a mediator in the function of the p53 gene, suppressing growth and promoting cell death [24]. Since all tumors, benign or malignant, are formed because of uncontrolled cell growth, a disturbance in tumor cell growth regulators is logically one of the main reasons for tumors being created. Molecules inhibiting growth are particularly important, because proteins prevent the amplification of cells containing a defective genome by inhibiting the cycle [25]. Two groups of genes are involved in carcinogenesis, namely oncogenes such as K-Ras and suppressor genes such as p53, APC, and DCC/DPC4, and all MMR genes; mutation occurs earlier in the APC and MMR genes in the process of developing a tumor, and p53 mutation is one of the final events in this pathway [26]. Such inhibitors include p53 and its associates, like the p21 gene. Studies on molecular mechanisms during the development process of cancers and technology progression are one of the reasons for valuing molecular markers [27]. Methods for assessing efficacy are available to researchers today, enabling them to quickly assess and confirm new genes, mRNA copies, and proteins. Differences in the expression of these molecules in healthy and malignant tissue allow the identification of genes and changes in pathways in human cancers. CDKs play an important role in the cell cycle. They are activated by positive regulators and inactivated by inhibitor factors [28]. Among these inhibitors, the p21 protein belongs to the Cip/Kip family and inhibits it by connecting to cyclin-CDK. The Walf/Cip1 gene on chromosome 6 encodes the p21 protein and is expressed in two pathways: dependent on and independent of p53. Its activity results in stopping the cell cycle, facilitating the repair of damaged DNA, a growth stop in the last stages of differentiation, being involved in the aging process of cells, and preventing apoptosis [29].

In this study, p21 was assessed in 15 healthy subjects and 15 esophageal cancer patients, and the results indicated no expression of this gene in the samples. Studies on similar samples showed an increased expression of p53 and cyclin D simultaneously in squamous esophageal
tissues. The association between these two genes is reviewed below. Since disorders in the \textit{p53} gene and protein are the most common problems in human tumors \cite{30} and \textit{p21} plays an important role in their activity pathway, the \textit{p53} gene can be cited as a key factor. Its mutation leads to the survival of the damaged cell and uncontrolled cell growth by allele deletion, gene mutation, or protein destruction \cite{31}. Although mutation in the \textit{p53} gene is accompanied by a concentration of \textit{p53} protein in tumor cells, it is not followed by \textit{p21} gene expression due to the nature of the protein \cite{29}. \textit{p21} protein, unlike \textit{p53}, is also considered as an antiapoptotic factor \cite{28}. Expression of this protein in normal and cancerous cells occurs in pathways dependent on and independent of \textit{p53}. There is no direct association between \textit{p53} and \textit{p21} in noncancerous tissues. In normal conditions, \textit{p21} is expressed in a pathway dependent on \textit{p53} in a cell, being amplified when exposed to mutagens or during the process of cell differentiation, and it inhibits DNA amplification by either inhibiting CDKs, interacting with core antigens related to amplification, or playing an important role in the final cell differentiation. Mutation in the \textit{p53} gene and abnormal function of its protein increase intracellular \textit{p53} protein, but cannot express the \textit{p21} protein \cite{29}.

Regarding the direct association between \textit{p53} and cell cycle regulation, it seems that \textit{CDKN1A} encodes \textit{p21} and regulates its transmission from different stages of the cell cycle. In most cells being reproduced, the \textit{p21} protein exists in low amounts. As \textit{p53} is activated by the breakage of DNA or matching intermediates, it connects to the motif linkage in the \textit{CDKN1A} promoter and increases transcription of the \textit{CDKN1A} gene significantly. In cancerous cells generated during mutations of \textit{p53}, however, the transcription of the \textit{CDKN1A} gene is distorted and thus fails to limit the cell cycle progression in response to damage to DNA and incomplete replicated chromosomes. In the cell cycle, the \textit{p53} protein affects \textit{p21} following stress and DNA damage, and it inhibits the cell cycle and suppresses tumor function.

In most cases, \textit{p53} protein tissue is found in its mutated form in tumors. Other factors may inhibit and prevent the expression of \textit{p21} in pathways other than \textit{p53} \cite{30}. However, recent data from both artificial and live tissue suggest that the \textit{p21} gene can be regulated independent of \textit{p53} \cite{32}. In cancerous tissues, \textit{p53} is mutated, the half-life of the mutated protein is increased, and the internal amounts of this protein increases, but it cannot express the \textit{p21} protein due to the lack of normal function. Regarding the path independent of \textit{p53}, studies have shown that the relationship between these two proteins is different depending on the cell type, tumor tissue, and different phases of differentiation \cite{29}. Thus, it seems essential to assess mechanisms of inhibition and activation while studying \textit{p21} to better understand tumorigenesis mechanisms. This may explain the variance in reports indicating the repetitive changes of \textit{p53} in ESCC, while the correlation between the mutation of \textit{p53} and the \textit{p21} protein in ESCC is controversial and needs further clarification \cite{33, 34}.

\textit{p21} generally inhibits gene amplification, but it can activate gene amplification under certain conditions. The role of \textit{p21} in improving inhibition of the cell cycle dependent on \textit{p53} and due to DNA damage is well defined \cite{35}. The basis for \textit{p21} activities in the nucleus and cytoplasm in certain situations can be studied as follows: \textit{p21} improves the kinase activity of CDK4/cyclin D or CDK6/cyclin D complex, and so controls improvement through $G_1$. \textit{p21} prevents CDK2-cyclin E, the phosphorylation dependent on CDK2 for retinoblastoma, and E2F1, thereby inhibiting gene amplification dependent on E2F1 and progression through the S phase. \textit{p21} prevents kinase activity of CDK2-cyclin A and CDK1-cyclin A, which are retrospectively required for progression through the S and $G_2$ phases. In addition, \textit{p21} prevents kinase activity of the CDK1-cyclin B1 and progression through $G_2$ and $G_2/M$. \textit{p21} also prevents the amplification of PCNA through the terminal carboxyl and thereby prevents the synthesis of DNA and modulates routes of DNA repair.

\textit{p21} can prevent the amplificatory activity of amplification factors E2F1, STAT, and Myc through a direct connection. Some antiapoptotic effects of \textit{p21} are thus explained which can
help its gene alteration activities. Phosphorylation of p21 in Thr145 prevents the amplification of nuclear p21 by downstream AKT1 activated by ERBB2 (a member of the epidermal growth factor receptor tyrosine kinase) or IKKβ [36–38]. Cytoplasmic p21 prevents apoptosis by preventing proteins involved in cell death. It is not clear whether phosphorylation of p21 by AKT1 or p21 alone acts in the cytoplasm or is required for cytoplasmic activity.

Genes inhibiting the mutated oncogene p53 and TGFβ pathways of tumors, both of which inhibit the growth of most cancer cells, include tumor-inhibiting genes. Inactivation of one of the tumor-inhibiting genes causes unbridled tumor cell amplification. Conversely, RAS and PI3K pathways, which are involved in increasing cell growth, include many proto-oncogenes that are often mutated. Oncogenes permanently activate growth stimulators. There are also pathways that include both cancer genes; in such pathways, the mutations that inactivate the tumor inhibitors and the mutations that activate the proto-oncogenes have paradoxical effects on the function of the pathway.

The cancer gene pathways play an important role in the discovery of cancer genes. Reports on overexpression of cyclin D in esophageal squamous cell cancer indicate that a high cyclin D level can be related to the upstream cancer gene pathways. The RAS pathway increases CCND1 expression, while WNT/APC inhibits CCND1 expression by inhibiting β-catenin. In addition, CCND1 is the direct target of activating the transcription dependent on c-Myc. The first known function of Myc is its ability to trigger cell division. Myc causes the expression of cyclin D and reduces the expression of p21. Myc is a strong proto-oncogene, and its expression increases in many cancers [39].

While the rise of unregulated p21 in cancers is often related to the reduction of the proliferative activity of p21 (including p53), upregulation or strengthening mutations in gene activity (that suppresses CDKNIA amplification) can contribute to the spread of cancer. For example, it is possible that suppressing CDKNIA amplification (the gene encoding p21 protein) affects the spread of cancer by Myc in cases where Myc expression is increased. This may be important in ERα-positive breast cancers; the upregulation of estrogen-dependent Myc and downregulation of p21 increase cell amplification, and failure in connection with Myc-p21 in resistance to antiestrogen treatment may also play a role [40].

Significantly, Myc proliferates AP4, an amplification factor that is increased in colony sample cells, colon, and rectum cancers, and can inhibit CDKNIA amplification [41]. AP4 significantly prevents inhibition of the cell cycle through p53, sensitzes cells to cell death induced by DNA damage, and can suppress TGFβ-dependent CDKNIA amplification [41]. The loss of inhibiting TGFβ growth activity is a signal of many cancers [42], so it seems that AP4 and other factors that prevent p21 expression and obstruct growth inhibition by TGFβ, such as miR-106b-25, a recently identified microRNA group [43], can help spread these cancers.

CDKNIA activation in response to a range of stimuli, including damage to DNA, depends on p53 and its co-member p73. CDKNIA amplification induced by H-RAS and BRCA1 mediated by mechanisms dependent on p53 and independent of p53 have also been shown. The activation of CDKNIA amplification is performed by growth factor, nuclear receptors, chemicals including anticancer agents (such as HDAC inhibitors), and drugs with antiproliferative activity (such as statins). Myc suppresses CDKNIA amplification by binding to Sp1 and preventing Sp1 [44], and this could be reduced by connecting the nuclear receptor hepatocyte nuclear factor of ligand 4α1 (HNF4α1) to Sp1 [45]. In response to damaged DNA, Myc is used to promote CDKNIA by MIZ-1 and form a triple complex of DNMT3a methyl transferase DNA that suppresses CDKNIA expression [46]. Moreover, AP4, a main helical ring/helical protein and the amplification target of Myc, suppresses CDKNIA through connectivity to four E-box motifs independent of MIZ-1, Sp1, or Sp3 [41]. The proliferative circuit of CDKNIA includes proliferative factors which prevent cell growth, differentiation, or senescence, and up- or downregulates CDKNIA expression under different circumstances. Some of these factors act...
in the proliferative networks, such as: adenomatous polyposis coli; C/EBPα (CCAAT/enhancer binding protein-α); CREBBP (CREB-binding protein); FGF2 (fibroblast growth factor 2); GAX or MOX2; HOXA10 (homeobox A10); IFNγ; IL-6; KLF4 (Krüppel-like factor 4); nerve growth factor; NRG1 (neuregulin); okadaic acid; phorbol-12-myristate 13-acetate; progesterone receptor; signal transducer and activator of transcription; TGFβ, and thrombopoietin [23].

Most studies on the role of p21 in cancer consider rat studies combined with factor analyses and biochemical methods in cell culture. The pioneering work that led to the early detection of p21 described the tumor-suppressing activity of p53 as a potential mediator [47]. Subsequent work showed that CDKNIA deletion in mice terminates a growth inhibition dependent on p53 and induced by DNA damage [48, 49]. Thus, p21 cannot explain the tumor-suppressing activities of p53; however, it is the main determinant of tumor protection by p53 [50] as CDKNIA deletion greatly accelerates tumor generation in mice in a state of mutated p53, which is unable to induce cell death, but maintains partial growth-inhibitory activity [51]. The first genetic evidence to confirm the tumor-suppressing activity of p21 originated from the discovery that CDKNIA developed spontaneous tumors [52]. Secondly, these tumors (mean age of 16 months), compared with those resulting from a deficiency in other tumor suppressor genes in mice, such as Trp53 [53], p16 [54], or Arf [55], suggest that a drop in CDKNIA itself is not enough for the deterioration of malignancy. Although many human cancers such as those of the colon and rectum, those in the area between the head and the chest, head and neck, and small lung cancers are related to the reduced form of p21, rare mutations resulting in the reduction of CDKNIA activity in human cancer [56, 57] indicate that p21 may not be a classic tumor suppressor. Instead, p21 has synergy with tumor suppressors and fights change in genes to protect against cancer. CDKNIA shortage with the participation of H-ras and Myc, the emergence of BCR-ABL1 [58], or with LNK4 deletion helps the progression of cell conversion and reproduction in culture. Together, these data are consistent with the theory of multistage tumor and the role of p21 in this process.

Proliferative regulation of p21 has been largely studied [59]. In cells without conversion or perishable duplication of chromosome, Ras gene change activates CDKNIA proliferation through two independent mechanisms, both related to and independent of p53. Independent replication of p53 needs the amplification factor of E2F1 for CDKNIA with activated Ras. E2F1 and E2F3 binding to the cis-active elements 119 and 16+ of CDKNIA strongly activate the proliferation of CDKNIA [59, 60]. Raf, a downstream effector of Ras, also exchanges CDKNIA independently of p53 [61]. Raf and Ras induce gene change and aging related to p21, and other genetic mutations that are important obstacles for the creation of tumor-like cell death are necessary to discard gene change-induced cell aging and death [62].

The important role of p21 in increasing H-ras-induced aging is confirmed by the finding that eliminating CDKNIA helps H-ras to activate tumor progression [63–65]. It is likely that the expression of genes involved in programming, such as SOX2 and OCT4, and the decreased expression of p53 and downstream objectives such as p21 and miR-145 are involved in cancer [25]. AMAPK acts in identifying cell stress. Energy shortage causes cell stress. By identifying the cell stress message and immigration to core, it is attached to protein p53, which turns off the tumor. Then, one phosphate connects to the histone near the p21 gene and causes the illumination of this gene. p21 activity delays the cell cycle or causes it to stop until the energy level of the cell has returned to normal.

It seems that viral factors can also abolish gene expression. Many viral proteins affect the stability or regulation of postproliferative p21 using the influence of cell proliferation. For example, the E6 protein of the human papillomavirus (HPV) can downregulate p21 independently of p53 [66, 67]. Although E6 is necessary for the genetic change of HPV and has antiapoptotic activity, it can downregulate p21 to enhance cell death [68] under some
circumstances, such as damaged DNA [66]. Virus type 2, associated with a tumor, preferably downregulates p21 protein in cells infected with HPV, with continuous increased activity of CDK2-cyclin E, but prevents the advance into the S phase; thus, it helps repeat tumor-associated virus type 2 [69]. The core protein of the hepatitis C virus prevents p21 postproliferatively, reduces inhibition of CDK2, and helps cause tumors by hepatitis C [70]. Finally, cyclin K encoded by herpesvirus 8 increases the phosphorylation of p21 in Ser130 by CDK6 without affecting its stability or cytoplasmic-nuclear position [71]. Interestingly, although the phosphorylation of p21 in Ser130 by CDK6 induces spread by E3 SCF SKP2 (SKP1-CUL1-SKP2) and breakdown, the phosphorylation of p21 by CDK6-cyclin K prevents the relationship of p21 with CDK2, thereby reducing the inhibition of phase G1 due to p21 [71]. The mechanism by which viral proteins affect the stability or activity of p21 is unknown. These findings suggest that p21 appearance is a common mechanism that regulates these viruses to cell cycle progression and cell death. RNA viruses as well as DNA viruses separately play important roles in the process of carcinogenesis by activating oncogenes and inactivating tumor suppressor genes, respectively.

An understanding of the role of p21 in suppressing tumors has been achieved from the study by Shen et al. [72], which shows a prominent tumor suppressor role for p21 regarding genomic instability. CDKN1A shortage along with a drop in ATM protein helps the development of chromosomal changes, which increases tumor progression [72]. Although the expansive malignancies of Trp53R172P+/+ keep stable genomes in mice, lymphoma and malignant tumors of connective tissue originate from Trp53R172P+/+; rat CDKN1A has an early invasion and shows particular chromosomal aberrations and changes in chromosome number [51]. The finding that downregulation of p21 is inversely correlated with microparasite instability in colon cancer, regardless of p53, confirms that the loss of protection against genomic instability by p21 helps malignant human conditions.

Some research suggests that the absence of p21 followed by an increase in the number of stem cells (such as keratinocytes) is strongly correlated with increased cancer ability [73–75]. Nevertheless, p21 has recently been shown to determine the maintenance of the self-replicating capacity of leukemic cells in rats with gene change PML PAR by protecting against deterioration caused by stressful conditions [76]. The results demonstrate that p21 is important for survival in at least a subset of malignancies. The results also show that this p21 activity can vary depending on a specific genetic change. Jaiswal et al. [77] investigated gene polymorphism of p21 at codon 31 and dinucleotide gene polymorphism of p73 regarding susceptibility to bladder cancer in northern India. The results showed that p73 gene polymorphisms are associated with susceptibility to bladder cancer, but no relationship exists between p21 gene polymorphism at codon 31 and susceptibility to bladder cancer. This result is consistent with the results obtained in the current study. Taghavi et al. [19] investigated p21 gene polymorphism and its relationship with smoking in ESCC in northeastern Iran. Their findings showed that none of the p21 genotypes were significantly associated with increased risk of ESCC, and polymorphisms of the p21 gene, alone or in combination, are not biomarkers of a genetic predisposition to ESCC. However, the presence of this polymorphism in combination with smoking was in contrast to the incidence of ESCC cancer in northeastern Iran [18]. This finding is consistent with the results obtained in the current study.

Villwock et al. [22] considered the prevalence of p21 immunohistochemistry in the epithelial tumor of the esophagus. Their results indicated that among 42 patients, 83.3% were male and older than 40 years. Surgical treatment was proposed to 56.2% of them: surgery of the whole stomach, or surgery of the stomach and transitional esophagus. Other patients underwent minor surgery or did not undergo any surgical treatment. Only 5 patients underwent chemotherapy and radiation, in combination and alone. Advanced diseases (stages 3 and 5) were detected in 78.6% of patients. According to the evaluation systems of
active immunity, only 9 patients were positive for p21. Ultimately, the researchers found that accumulation of p21 did not play a key role in glandular epithelial esophageal cancers in the patient population [22]. This result is consistent with the results obtained in the current study.

Liu et al. [75] investigated the relationship between the state and prognosis of p21\textsuperscript{WAF1} in esophageal squamous cell cancer stage 3. The results showed that the overall survival rate for the p21\textsuperscript{WAF1}-positive group was significantly higher than that of the p21\textsuperscript{WAF1}-negative group. No significant correlation was observed between p21\textsuperscript{WAF1} and the clinical pathology parameters according to sex, age, tumor location, tumor grade, pathological stage, and number of lymph node invasions. They concluded that p21\textsuperscript{WAF1} plays a complex role in tumor genesis and development of ESCC. p21\textsuperscript{WAF1} can be used as a prognosis predictor for patients with ESCC stage 3. This result is consistent with the results obtained in the current study.

Ishida et al. [76] examined the existence of p53 and p21 and the clinical response to temperature shock treatment by radiochemotherapy in patients with ESCC. Multivariate analysis revealed that the existence of p21 is an independent risk factor related to histological effects. None of the 10 patients with negative p21 and positive p53 tumors in their study showed a third-grade response, while the other 55% combined with the other group did. They concluded that the combination of p21 and p53 in histological findings can predict hyperthermochemoradiotherapy histological function.

Regulation of p21 transcription is widely studied [59]. Positive expression of p21 is associated with disease progression and poor prognosis in prostate [80], ovarian, and breast cancers [81]. Yet, given the frequent increased expression of p21 in human cancers, understanding the mechanism of action of this gene offers considerable insight into the development of human cancers. The inhibition of p21 is in negative correlation with microsatellite instability in colon cancer, regardless of the status of p53; thus, it helps the loss of protection against genomic instability of p21 to human tumors [82]. Studies have demonstrated a reduction in the amount of p21 in verrucous leukoplakia of the mouth, in the incidence of p21 in muco-epidermoid carcinoma of the salivary glands, and in oral ameloblastoma [25]. Studies on colorectal tissue showed the important role of p53 gene mutation in the development of colorectal adenocarcinoma and strengthened the theory of expression of p21 in pathways independent of p53 in colorectal cancer [83]. The amount of expressed p21 is decreased to a greater extent in tumor cells than in nontumorous colons of patients and nonpatients, but it is not related to prognosis.

Differences in the amounts of reported p21 gene mutations in different areas reveal that the incidence of mutation in this gene is strongly influenced by environmental, geographic, and ethnic factors. However, the type of test and the technique used to detect mutation are also very important. The p21 protein is one of the best known proteins related to the cell cycle. Reduction or loss of expression of the p21 protein indicates a poor prognosis in these cancers. As p21 is a prognostic marker and its nonexpression will cause cell amplification, it seems normal that well-differentiated tumors express this marker more and tumors with a higher microscopic stage and lower differentiation express this marker less. This is exactly what the findings of most studies, including the current one, support. As p21 is a prognostic marker and stage has a greater value than grade, the significant relationship between them is rational. Unfortunately, they were not assessed in the current study because of the aforementioned restrictions.

There is an inverse relationship between the prevalence of the expression of the p21 protein, phase of tumor, depth of invasion, and lymph metastasis in colorectal cancer, and the prognosis and survival are desirable in the presence of these proteins [11]. Apparently, the reasons why a lack of gene expression and significant differences were obtained were that the tumor samples assessed were in stages 4 and 5, gene expression faces changes in the early
stages of cancer, genes express in the early stages of cell cycle inhibition, and they do not express in the later stages of cancer.

Reduced expression of P21 is associated with an increase in liver and lymph involvement. It is also accompanied by deeper tumor invasion into the muscle layers and a higher stage, which confirms the theory of the current study. The biological activity and network-regulatory activity of P21 complex make predicting its function in treating cancer possible. Different effects of P21 on gene regulation and its role in genomic stability, cell death, aging, and DNA repair may not only help cancer development, but also largely affect the benefit of agents damaging DNA or other drugs that can induce P21. Different anti-cancer agents such as HDAC inhibitors act through their ability to enhance the induction of P21, at least in part [81]. Other factors such as statins (commonly used to decrease cholesterol levels) show a profound antiproliferative capacity by inducing P21 and are being investigated for their anti-tumorigenesis activity [82]. The only challenges are in the selective inhibition of the gene-changing activity of P21 and tumor suppression activities. Therefore, the development of agents that inhibit the ability of P21 to integrate CDK4-D-cyclin and CDK6-D-cyclin complexes and its ability to suppress CDK1 or CDK2 can be an interesting line of study. Likewise, instead of P21 markup, selective marking of upstream and downstream factors of P21 that affect the specific aspects of its activity is beneficial. Drugs that can block the anti-apoptotic activities of P21 are probably particularly effective when combined with other drugs that can induce P21, such as agents that damage DNA. Remarkable progress has been newly achieved. Structural and biochemical studies of different ligase complexes directly involved in the analysis of P21 protein under different conditions will undoubtedly help the development of selective inhibitors for these ligases and provide guidance on how to develop a new generation of anti-cancer agents. In addition, DNA-damaging agents may selectively inhibit the activity of AKT1, may not only prohibit tumors from AKT1 activities or possibly unstable P21, and may lead to an increase in their apoptotic effects. It is likely to be confirmed by research that the DNA-damaging agent aminoflavin can induce cell death of MCF7 breast cancer cells only at concentrations that reduce the activity of AKT1 and unstable P21. Alternative treatment methods may be replaced using the ability of P21 to induce senescence in tumors. Recent work suggests that tumor regression can be achieved through the reactivation of aging, for example by restoring P53 activity or disabling Myc in tumors with P53 [83]. Despite the inactivation of Myc from P21 with up-regulation in a subset of tumors, the results demonstrate that the activation of aging is not only possible, but is also a promising approach to regressing tumors in living tissue. Even in tumors with high amounts of P21, tumor regression through the reactivation of aging is possible. Nevertheless, this possibility requires a greater understanding of the various factors that can destroy P21-induced aging despite high levels of P21. The appearance of P21 in these tumors can potentially be used for treatment by marking ID1 or similar molecules that will lead to the reactivation of the aging process of P21. Finally, the need for scientific progress to identify the exact role of P21 in modulating DNA repair processes under different conditions is strongly sensed and may lead to new strategies of the role of P21 in the development and treatment of cancer [23]. Expression of P21 gene by the adenovirus vector was able to reduce cell growth. Cell growth inhibition was associated with the induction of a squamous cell marker. The researchers performed P21 gene therapy using a gene gun technology. Gene delivery, using a gene gun, uses a shock wave to accelerate the entry of micro-particles covered with the plasmid to the target cell or tissue. Since this method is not associated with cell surface receptors, it can introduce genes into a wide range of mammalian cells. The gene gun is simpler and less aggressive than other forms of gene delivery. Significant inhibition of growth was observed in cells that did not express P21 after this method was used. The impact of P21 protein on the treatment varies depending on the expression direction, and many consider its activity with higher sensitivity to chemo- and radiotherapy in the presence of the
P53 natural protein. Analysis of gene expression may be useful in providing information on the factors affecting prognosis. One of the best functions of using real-time PCR is gene expression assessment using relative quantification; this method is the most accurate way to detect changes in gene expression. In this method, only an increase or decrease in gene expression is important, and the increase or decrease is compared with the standard, the internal control. This method is cost-effective economically and in terms of time [84]. The advantage of real-time PCR is the use of a reference gene, which is continually expressed in all cells under investigation; a detailed assessment can be carried out with this gene despite the changes in RNA quality and efficiency of each reaction. The reference gene should be expressed in all samples at the same level; otherwise, the normal process will result in errors in the results. Test method and identification technique are important. Different immunohistochemical studies analyzed P21 in a variety of tumors. Some studies reported a relationship between P21 status and worse prognosis in breast [14], anal [15], bladder [16], and ovarian cancer [17] and poor prognosis in prostate cancer [77]. They have also observed no significant difference in esophageal cancer (adenocarcinoma). However, no research has been conducted on this gene and these cancers using real-time qPCR. This study investigated the expression of P21 by real-time qPCR for the first time in Iran. It is hoped that this study will be a prelude to designing prospective cohort studies with patient follow-up, investigating survival status, recurrence, response to treatment, and determination of relationships with other genes involved in esophageal cancer.

Conclusions

The results indicated no significant difference in p21 gene expression between patients and controls (p > 0.05). Moreover, no significant difference was observed in p21 gene expression between males and females (p > 0.05). It appears that p21 is not a specific tumor marker in Iranian esophageal cancer patients.

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Statement of Ethics

The authors declare that there are no ethical issues in this article including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.

Disclosure Statement

The authors have no affiliation or financial involvement with organizations or entities with a direct financial interest in the subject matter or materials discussed in this article.
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