

Original Article

Expression of *CXCL5* Gene in Patients Suffering from Non-Small Cell Lung Cancer

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Abstract

Introduction: Lung cancer is one of the most common cancers with high mortality rate because of the late diagnosis. The present study aimed to quantitatively measure the C-X-C Motif Chemokine Ligand 5 (*CXCL5*) gene expression level in tissue samples of lung cancer patients to investigate its value as a biomarker during lung cancer diagnosis and screening.

Materials and Methods: Tissue samples were collected from 30 patients. Total RNA was extracted from tumor and normal tissues of patients. The rate of *CXCL5* gene expression was initially measured in A549 cell line and next, the expression level of this gene in tumor tissue samples of patients suffering from Non-Small Cell Lung Cancer was compared to the normal lung tissue of the same patients.

Results: The results demonstrated significant increase of *CXCL5* gene expression in cancer samples compared to normal tissues of the same samples. The increase was 5.8 fold for cancerous tissues in comparison with normal tissues ($P=0.03$). There was no difference between the tumor type (adenocarcinoma, squamous cell carcinoma) and average *CXCL5* gene expression rate ($P=0.09$). In cancerous samples, the expression level of *CXCL5* was higher in men compared with those of women ($P=0.04$). There was no relationship between the change of gene expression and the age of the patients ($p=0.08$).

Conclusion: Based on the results, it can be concluded that the quantitative expression level of *CXCL5* in lung cancer patients could be used as a biomarker to screen lung cancer samples, regardless of age of patients and tumor type. However, it can discriminate the stage of tumor.

Keywords: Lung Cancer, *CXCL5*, Real-time PCR

1. Introduction

Lung cancer is regarded as the most common cancer worldwide. Statistics shows that the mortality rate of breast, prostate, and intestinal cancers altogether are less than the mortality of lung cancer [1]. More than 90% of lung cancer cases are issued by changes in lung basal epithelial cells and lung covering layer [2]. Lung epithelial cancers have four primary cell types,

including small cell lung cancer (SCLC) and another group called non-small cell lung cancer (NSCLC), consisting of adenocarcinoma, squamous cell carcinoma (SCC) and large cell carcinoma [3].

One of the genes which expresses changes during the progression of lung cancer is *CXCL5* (C-X-C Motif Chemokine Ligand 5 of neutrophil-activating epithelial peptide). It belongs to CXC-type chemokine family,

encoding small secretory proteins. The product of *CXCL5* acts as an angiogenic chemokine which is considered as an inflammatory mediator and a potent absorber of neutrophils. There are some evidence about the role of CXCL in carcinogenesis and cancer progression [4]; for instance, the increase of *CXCL5* expression is observed in colorectal [5], prostate [6], endometrial [7], squamous cell and pancreatic cancers [8]. The expression is associated with advanced stages of tumor, local invasion and metastasis. These studies revealed that *CXCL5* directly stimulates cancer cell proliferation. Besides, *CXCL5* directly causes endothelial cell proliferation and invasion *in vitro*, and also increases tumor angiogenesis in non-small cell lung carcinoma and pancreatic cancer [9].

CXCL5 gene is also called epithelial cell-derived neutrophil-activating peptide ENA-78 and is expressed by epithelial cells after stimulation by pro-inflammatory cytokines such as IL-1 β and TNF α , which in turn, results in the absorption of polymorphonuclear neutrophils (PMN) [10]. On the other hand, the combination of HB-EGF (EGF-like growth factor binding to heparin) and mucosal neutrophils (activating peptide-78) makes a strong synergistic effect on cancer proliferation, epithelial-mesenchymal transition, migration and invasion. *CXCL5* not only stimulates the classic pathway of EGFR and the signaling pathway AKT and ERK/RSK1/2, but also increases heat shock protein phosphorylation (HSP27) [6].

CXCL5 is produced by different kinds of cells such as monocytes, neutrophilic dendritic cells, fibroblasts and mucosal cells. These cells involve in regulating the biological processes of cancer, including the induction of tumor angiogenesis by proliferation, endothelial cell invasion, mobility, invasion and tumor metastasis. Recent studies have indicated that *CXCL5* neutralization by antibody has a significant role in the incidence of cancer progression [11]. Some studies have suggested that

CXCL5 is also released through stimulated endothelial cells in the lung and other human tissues and can cause the creation of pro-adhesive activity of neutrophils along with IL-8 [12].

Some researchers used the assessment of *CXCL5* chemokine level as a biomarker for the diagnosis of prostate cancer. They stated that alteration of the gene expression was one of the primary causes of the prostate cancer initiation and by increasing its expression, the prognosis of the disease would get worse [13]. In another research assessing the expression level of chemokine showed to be a good predictor for cortical cancer prognosis [5]. Through using ELISA and the immunohistochemistry methods, it was shown that the expression level of this chemokine in tumor tissues was significantly higher than normal tissues. Therefore, it is applied as a biomarker to predict the diseases and determine the patient's prognosis [14].

The possibility of using *CXCL5* level assessment in the early diagnosis of lung cancer was proposed by Kowalczyk et al. They compared the expression of 18 different genes in normal and cancerous tissues. The results demonstrated that a significant difference existed between the normal and cancerous tissues for *CXCL5* gene expression [15].

Since lung cancer is one of the major causes of global death and its 5-year survival rate is lower than other cancers [1], it is necessary to develop methods and tools leading to the early diagnosis of the disease and hence, the mortality reduction. To achieve this aim, the present study was intended to measure *CXCL5* expression rate of patients suffering from NSCLC and assess whether it is a suitable biomarker to predict the stage of the cancer.

2. Materials and Methods

2.1 Sampling

The present study was designed and conducted in accordance with the Helsinki declaration and relevant guidelines. Ethical

approval for this project was obtained from ethics committee of Islamic Azad University of Tehran with a code of 340/22. In this study, 30 patients were selected after being diagnosed by the specialist and participated in current research after completing the informed consent. The questionnaire and satisfaction letter was prepared as stated in the ethical rules in the Experimental Medical Studies of Helsinki. The samples were lung tissue biopsies. From each patient diagnosed with NSCLC, 2 biopsies were taken: one from normal lung tissue and the other from the tumor (in total 60 samples). They were collected through bronchoscopy method by the specialists from patients admitted to Shahid Masih Daneshvari Hospital. These biopsies have been gathered regardless of age, gender and clinicopathological data. After collecting, the tissue samples were transferred to the nitrogen tank and were preserved in -80°C freezer in accordance with principles of transferring and maintaining. All ethical guidelines as of keeping and using human samples were granted.

2.2 Expression Analysis

CXCL5 gene expression was measured in the patients' tumor tissue samples and their normal lung tissues. Moreover, the beta-actin (*ACTB*) gene was considered as the control or reference gene for measuring the results. Initially, *CXCL5* gene expression rate was evaluated in A549 cell line; later, the expression level of this gene was compared in tumorous and healthy tissue samples of patients suffering from NSCLC.

Total RNA extraction was performed from both A549 cell line and tissue samples (tumor and healthy) by GeneAll kit. After RNA extraction, its quality and quantity were evaluated through using horizontal gel electrophoresis on a 1% agarose gel and noanodrop.

To synthesize cDNA, samples with absorbance ratio 260/280 nm between 1.8-2.0 were applied. 1 μl of RNA was used in the Applied Biosystem (ABI). Revert Aid First Strand cDNA Synthesis Kit (Fermentase) was used to produce cDNA. The primers were designed by Primer Express software version 3 (Applied Biosystems, Austin, TX, USA). The primers are presented in Table 1. The sequence of *ACTB* was used as internal control.

The total reaction components in real-time PCR reaction consisted of 12 μl Syber TM Master mix, 1 μl forward and reverse primers, 2 μl cDNA and 9 μl sterilized distilled water. Real Time PCR was performed for *CXCL5* and *ACTB*, using 480 Master mix, SYBR Green I (Roche Applied Science) with thermal program as follows: initial denaturation at 95°C for 4 minutes, the second stage over 35 cycles at 95°C for 45 seconds, 57°C for 30 seconds and 72°C for 30 seconds. Fluorescence ratio was assessed by Exicycler 96 TM. (Bioneer, South Korea). Reproduction and separation curves were plotted and analyzed by software. In order to confirm the amplification of components and correctness of PCR reaction, the product of Real Time PCR reaction was measured by nanodrop device and on a 2% agarose gel. The results of confirmation on agarose gel are presented in Figure 1. Besides, the results of the cycle of *CXCL5* and *ACTB* gene expression amplifications are plotted in Figure 2.

Raw data of Real Time PCR was analyzed by REST2009 software. All the tests were performed in triplicate. The cyclic threshold (CT) of *CXCL5* and housekeeping gene (*ACTB*) for each sample was determined. The ΔCT was calculated by using the CT of *CXCL5* and *ACTB* to normalize the data. Finally, according to Relative Quantization and $2^{-\Delta\Delta\text{CT}}$ formula, the fold change was determined for cancerous and normal tissues.

Table 1. Nucleotide sequences of primers for CXCL5 and ACTB genes

Primer designation	Primer sequence (5'.....3')
<i>CXCL5</i>	F: TCGTCCGCGGAGCTATTCG
	R: CTCCGGTGCACGCCGAGCC
<i>ACTB</i>	F: TCCTCCTGAGCGCAAGTAC
	R: CCTGCTTGCTGACCACATCT

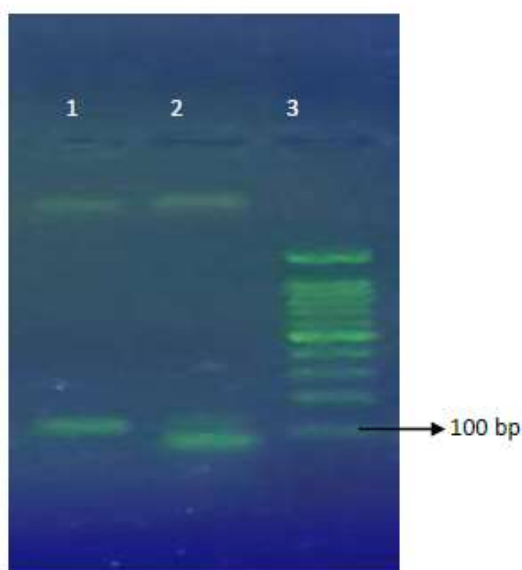


Figure 1. Well 1 contains the PCR product of *CXCL5* gene, with the length of 126 bp. Well 2 contains the product of *ACTB* gene. The length of the fragment is 88 bp. Well 3 presents the DNA marker.

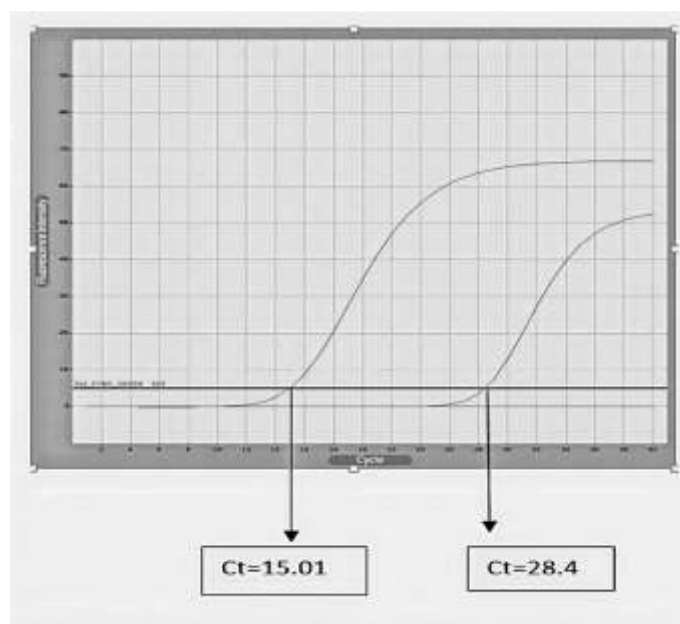


Figure 2. Amplification of *ACTB* and *CXCL5* genes. The vertical axis represents fluorescence amount and the horizontal axis shows the number of cycles. The amount of CT for the *CXCL5* was 28.4 and for the *ACTB* was 15.01.

2.3 Statistical Analysis

All statistical analyses were performed by SPSS version 20. Pearson's chi-squared test and independent t-test were used to analyze categorical and continuous variables, respectively. P-value < 0.05 was considered statistically significant.

3. Results

Participants of this study were comprised of two groups of lung tissue types (tumor and normal), in which 30 individuals were included. 18 subjects (60%) were male and 11 subjects (40%) were female. The mean age of patients was 65.4 ± 0.883 . Thus, it can be argued that age factor has no disturbing effect over the studied groups. In addition, with a similar comparison made in the subgroups of men and women, there is still no difference in the average age of these subgroups as mentioned previously in this study. In the current research, the samples were divided into four stages

according to the state of tumor, which included the samples of the first tumor stage (6 samples (20%)), second tumor stage (12 samples (40%)), third tumor stage (5 samples (16.7%)) and fourth tumor stage (7 samples (23.3%)).

The results obtained from real time PCR, shown in figure 3 revealed that the relative alteration of *CXCL5* gene expression in all cancerous samples (without considering the stage of tumors) was 2.5 fold more than its expression in normal samples and their difference was statistically significant ($P < 0.05$). There was no difference between the tumor type (adenocarcinoma, SCC) and average *CXCL5* gene expression ($P = 0.09$); however, the stage of cancer was significantly effective in the change of given gene expression ($P = 0.03$). For instance, 5.8-fold increase was detected in stage IV tumor tissue (Figure 3).

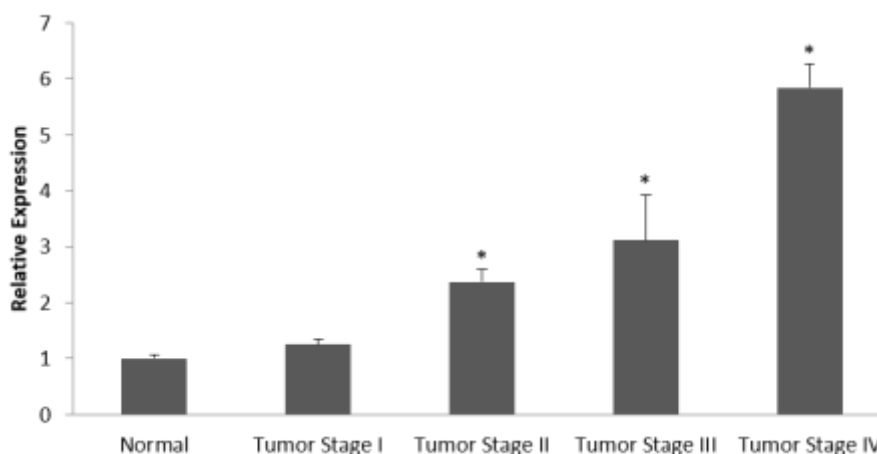


Figure 3. Relative expression of *CXCL5* in different stages of NSCLC. A significant difference ($P = 0.03$) of *CXCL5* expression in tumors in various stages of NSCLC and normal tissue was identified. For tumor at stage I, II, III and IV the measured expression was 1.25 ± 0.1 , 2.37 ± 0.23 , 3.12 ± 0.08 and 5.85 ± 0.42 , respectively.

In the case of the gender, the expression rate of this gene in women was lower than men and this difference was statistically significant ($P = 0.04$) (figure 4). The significance of this issue is determined when it is necessary to measure the

expression for diagnosis of the disease. Consequently, the cut off value specified in the diagnosis should be different between men and women. This study revealed that no relation exists between the expression changes of *CXCL5* and the age of the

patients ($P = 0.08$). One fascinating finding of current study was the difference

of *CXCL5* expression levels between smokers and non-smokers (figure 5).

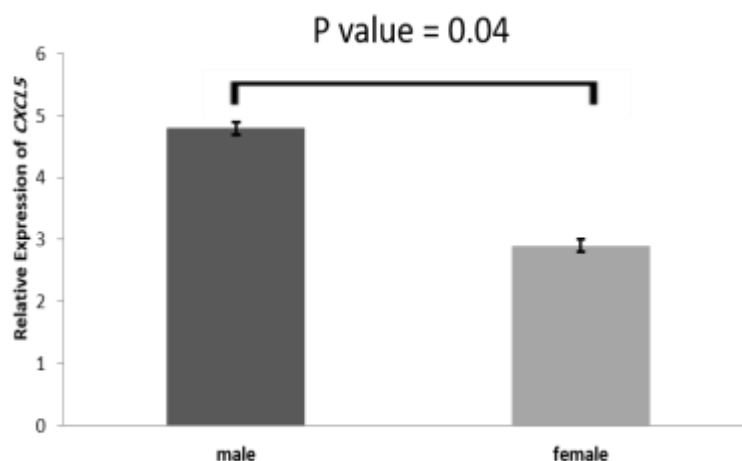


Figure 4. Relative expression of *CXCL5* in patients of both genders. A significant difference ($P= 0.04$) has been observed. The level of expression in female and male was 2.9 ± 0.66 and 4.8 ± 0.74 , respectively.

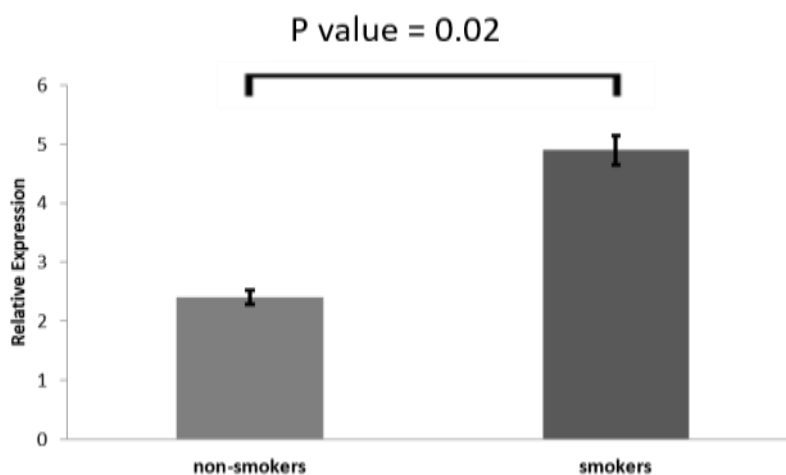


Figure 5. Comparison of *CXCL5* relative expression between smokers and non-smokers. In non-smoker patients the average of expression was 2.4 ± 0.37 ; in smokers it was 4.9 ± 0.42 .

4. Discussion

The results of the current study exhibited significant elevation of *CXCL5* gene expression at 5.8 times in NSCLC biopsies compared to normal biopsies of the same patients. The results showed a substantial link between the degree and severity of cancer and increase the expression of *CXCL5*. Therefore, evaluation of this gene

expression can be suggested as an identifying biomarker for NSCLC.

The results of the present study were consistent with the results achieved by Pold et al. [16] concerning the increased *CXCL5* expression in cancerous tissues. However, in the study carried out by Pold and his colleagues, *CXCL5* gene expression in A549 and H157 lines with cyclooxygenase 2 was assessed and indeed, the study of this

group on *CXCL5* expression was more comprehensive due to finding a way to treat lung cancer through this gene [16]. Their results revealed that one of the main reasons for the progression of cancer is its ability of angiogenesis. As mentioned earlier, *CXCL5* is one of the major angiogenic factors in the process of angiogenesis in cancerous tissues [17]. Hence, they sought to reduce the expression of this gene through inhibiting the expression of cyclooxygenase 2 so that in this way, they can prevent the progression of lung cancer [16] while the present study aimed to measure the expression of this gene to diagnose the disease and establish a relationship its expression rate and tumor size. The results of current research demonstrated that *CXCL5* gene expression rate in the cancerous tissue and cancerous cell line under investigation became 5.8 times higher compared to the control group and the statistical analysis also showed that this difference is quite significant ($P<0.05$). Therefore, it assumed that finding an appropriate cut off, it can be used as a diagnostic biomarker in disease screening. However, other studies have been conducted regarding the measurement of *CXCL5* expression as a factor in the diagnosis of cancer, including the research of Zhu et al. [18]. In their investigation, the measurement of *CXCL5* expression was used as a factor in the diagnosis of the bladder cancer. It was similar to this study in that they also used the measurement of *CXCL5* gene expression in the tissue and cell line. The results of their research on the cell line indicated that the increased expression of the aforementioned gene caused increase in the proliferation and progression of the tumor cells, and expression of this gene in the tissue suggests its increased expression relative to the normal tissue, which was congruent with the results of the present study on the lung cancer tissue. Another result obtained by this group showed the significant relationship between the expression rate and

cancer stage ($P<0.05$) [19]. The relationship between *CXCL5* gene expression and disease stage was also evaluated in our study and the result of statistical analysis revealed that *CXCL5* gene expression significantly enhances with the disease progression ($P=0.03$) (Figure 2). This study's results were consistent with the findings achieved by Li et al. [8], in which *CXCL5* gene expression pattern pancreatic cancer was obtained. They demonstrated that the expression rate of the mentioned gene was raised by the disease progression. In addition, the expression rate in men was significantly higher than in women ($P<0.04$) [8]. It seems that by the inhibition of *CXCL5* gene expression, lung cancer can be treated. Until now, this issue has been proved in some investigations. Miyazaki et al. stated that by decreasing the expression of this gene through the RNA interference technique, the proliferation ability and invasion of cancer cells in the culture medium can be reduced [19]. Concerning the role of chemicals including cigarette in the incidence of lung cancer, the role of cigarette in increasing *CXCL5* gene expression was examined in the present study and the results suggested that among smokers, *CXCL5* expression level is almost 2.5 times higher than that of non-smokers ($P<0.05$) (Figure 3).

This point has also been proven in other studies [20, 21]. In one research, the relationship between smoking and the function of *CXCL5* factors and the inflammation was investigated among rats. The findings revealed that the rats in which *CXCL5* gene expression had been inhibited, lower response to smoke was observed [22]. This could be the approval for the results of the present study, indicating the increase of *CXCL5* expression in smokers. Hence, to diagnose cancer in smokers, a higher level of *CXCL5* gene expression rate should be considered as alarming. Since the purpose of this study was to use the findings in clinical examinations for the diagnosis and

treatment of lung cancer, all the factors affecting the expression of CXCL5 gene should be considered. Another aspect was analyzing of the gene expression and the tumor type. The expression level was compared in adenocarcinoma and SCC and it was found that there is no significant difference among the types of lung tumors in terms of CXCL5 gene expression. Accordingly, measurement of this factor cannot be helpful in the diagnosis of the tumor type. Considering the results obtained from this research and similar studies, it can be concluded that this factor can be an appropriate diagnostic biomarker for the early diagnosis of the disease and may be used in screening the patients at risk through further investigations. In the current research, the expression level of CXCL5 was measured in biopsy samples to investigate its amount in normal and cancerous biopsies. It should be noted that among non-invasive methods, tumor biomarkers that are produced in response to the presence or progression of cancer from the body or tumor tissue and are found greatly in blood, urine and tumor tissue compared to healthy people are proper criteria for the early diagnosis of the disease [23].

At the end, this project was done considering the importance of finding new biomarkers to non-invasively diagnose NSCLC in people who are at risk. It could be better to repeat this investigation on a large scale population to achieve more precise results and cut offs. For instance, in smokers, the results showed there should be a higher cut off in comparison to non-smokers. It is better to consider the disease or situation in which people have higher level of CXCL5, because this can eliminate the probability of errors. Previous studies showed the elevation of CXCL5 protein in multiple cancers [9, 24], so evaluation of mRNA level of this gene is cost-effective and reduces the expenses of test.

5. Conclusion

Results obtained from this research and similar studies suggest that the CXCL5 mRNA level in cancerous tissues increases drastically. It could be considered as an appropriate biomarker for early diagnosis of different kinds of NSCLC. As its expression is significantly and continuously elevated during progression of NSCLC, it can be used as a discriminative biomarker to categorize various stages of the tumor. In addition, it can be a proper tool to screen at-risk people.

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Conflict of interest

The authors declare no conflict of interest.

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