Research Article / Araştırma Makalesi

Elevated Serum Levels of Inducible Nitric Oxide Synthase, Monocyte Chemoattractant Protein-1, and Cyclooxygenase-2 In Patients with Lung Cancer

Akciğer Kanserli Hastalarda İndüklenebilir Nitrik Oksit Sentaz, Monosit Kemoatraktan Protein-1 ve Siklooksijenaz-2'nin Yüksek Serum Düzeyleri

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Abstract: Lung cancer is a malignant lung tumor characterized by uncontrolled cell growth in lung tissue. Genetic and epigenetic abnormalities can be seen in lung cancer. These abnormalities can lead to activation of oncogene and inactivation of tumor suppressor genes. Inflammation is a powerful mediator of cancer development. Pulmonary inflammation may play a role in the initiation or progression of cancer. The main mediator of inflammation is inducible nitric oxide synthase (iNOS), which synthesizes nitric oxide from L-arginine. Monocyte chemoattractant protein-1 (MCP-1) is one of the important chemokines that regulate the migration and infiltration of monocytes/macrophages. It has been determined that MCP-1 plays an important role in lung allergic inflammation, lung leukocyte infiltration and bronchial hyperresponsiveness in the pathogenesis of asthma. Cyclooxygenases (COX) are responsible for prostaglandin production from arachidonic acid. They contribute to inflammation-induced carcinogenesis. COX2 is the enzyme responsible for inflammation induced by inflammatory stimuli, hormones and growth factors.
In line with the information given, in this study, serum levels of COX2, iNOS and MCP-1 were determined using the ELISA method in 90 (36 adenocarcinoma, 36 squamous cell, 18 small cell carcinoma) lung cancer patients and 90 healthy control individuals. It was determined that COX2, iNOS and MCP-1 serum concentrations in lung cancer patients were significantly higher than in control individuals ($p<0.001$). However, no statistically significant difference was detected between lung cancer histological subtypes ($p>0.05$). It is thought that our findings may contribute to early diagnosis and development of new treatments for lung cancer.
Keywords: Lung Cancer, Inflammation, iNOS, MCP-1, COX2

Özet: Akciğer kanseri, akciğer dokusunda kontrolsüz hücre büyümesi ile karakterize kötü huylu bir akciğer tümörüdür. Akciğer kanserinde genetik ve epigenetik anormallikler görülebilir. Bu anormallikler, onkogenin aktivasyonuna ve tümör supresör genlerin inaktivasyonuna neden olabilir. İnflamasyon kanser gelişiminde güçlü bir aracıdır, tümörün büyümesine ve vücuda yayılmasına yardımcı olur. İnflamatuar hücrelerden salınan reaktif azot veya oksijen türleri DNA'ya bağlanabilir ve genetik değişikliklere neden olabilir. Böylece, pulmoner inflamasyon kanserin başlamasında veya ilerlemesinde rol oynayabilir. İnflamasyonun ana aracısı, nitrik oksidi L-arginin'den sentezlenen indüklenebilir nitrik oksit sentazdır (iNOS). iNOS'un tümör ilerlemesindeki rolü çok karmaşıktır, hem promotör hem de inhibitör etkiler belirlenmiştir. Akut ve kronik inflamatuar reaksiyonlarda lökosit göçü ve kemokinlerin aktivasyonu önemli fonksiyonlardır. Monosit kemoatraktan protein-1 (MCP-1), monosit/makrofajların migrasyonunu ve infiltrasyonunu düzenleyen önemli kemokinlerden biridir. MCP-1'in astım patogenezinde akciğer allerjik inflamasyonu, akciğer lökosit infiltrasyonu, bronş aşırı duyarlılığı ve eozinofillerin alınmasında önemli rol oynadığı tespit edilmiştir. Siklooksijenazlar (COX), araşidonik asitten prostaglandin üretiminden sorumludur. Prostaglandinlerin sentezi inflamatuar bölgede önemli ölçüde artar. Ayrıca, inflamasyona bağlı karsinogeneze katkıda bulunurlar. Siklooksijenaz 2 (COX2), inflamasyon uyaranları, hormonlar ve büyüme faktörleri tarafından indüklenen inflamasyondan sorumlu olan enzimdir. Verilen bilgiler doğrultusunda bu çalışmada, COX2, iNOS ve MCP-1'in serum seviyeleri 90 (36 adenokarsinom, 36 skuamöz hücreli, 18 küçük hücreli karsinom) akciğer kanseri hastası ve 90 sağlıklı kontrol bireyde ELISA yöntemi kullanılarak belirlendi. Akciğer kanserli hastalarda COX2, iNOS ve MCP-1 serum konsantrasyonlarının kontrol bireylere göre anlamlı derecede yüksek olduğu belirlenmiştir ($p<0.001$). Ancak akciğer kanseri histolojik alt tipleri arasında istatistiksel olarak anlamlı fark tespit edilememiştir ($p>0.05$). Bulgularımızın akciğer kanserinde erken teşhise ve yeni tedavilerin geliştirilmesine katkıda bulunabileceği düşünülmektedir.
Anahtar Kelimeler: Akciğer Kanseri, İnflamasyon, iNOS, MCP-1, COX2

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1. Introduction

The most prevalent form of cancer in the world, lung cancer has a high prevalence and is the main factor in both men's and women's cancer-related deaths. It is acknowledged that smoking, asbestos, radon, chromium, nickel, cadmium, and cobalt exposure at work and in the environment as well as a family history of the disease all contribute to its genesis. Over the past ten years, significant advancements in our knowledge of the genetics of lung cancer, the immune system's role in the prevention of lung cancer, and lung cancer therapy choices have led to significant improvements in lung cancer epidemiology and prevention. Nevertheless, despite these advancements, lung cancer continues to be the leading cause of cancer deaths and will continue to be a serious health issue that will worsen globally in the years to come due to smoking's rising prevalence on a global scale as well as exposure to environmental and industrial carcinogens (1, 2).

Squamous cell carcinoma, adenocarcinoma, small cell carcinoma (SCLC), and large cell carcinoma are the four main histological forms of lung cancer. The majority of lung carcinomas, or about 80% of them, are non-small cell lung cancers (NSCLC) (3, 4). Innate and adaptive immune cells become active when there is inflammation, which is an old, developed process. Inflammation plays a crucial function in host defense against pathogens, but it is also crucial for tissue remodeling, repair, and regeneration. It is also necessary for the regulation of tissue homeostasis. Inflammation and the immune system play a role in the onset, spread, and management of cancer, according to current research. Tumor growth and the development of cancer are both aided by inflammation. The inflammatory tumor microenvironment is created when cancer cells interact with the stromal and inflammatory cells nearby (TME). The phenotypic and functional characteristics of TMJ cells can alter often (5).

One of the hallmarks of cancer is inflammation. COX2 (cyclooxygenase-2) is a significant role in regulating the progression of cancer, including colon cancer, stomach cancer, esophageal cancer, lung cancer, breast cancer, and skin cancer. COX2 is a key inflammatory component. It has also been demonstrated that aspirin can slow tumor growth by inhibiting COX2 expression. Through a variety of molecular processes, COX2 regulates tumor growth as a significant biological component. Prostaglandin E2 (PGE2), which is produced as a result, is catalyzed and promotes the EGFR-ERK pathway, hence promoting tumor growth. By increasing the production of BCL-2 and decreasing the cleavage of caspases, it can also prevent tumor cells from dying. Additionally, via regulating neutrophil infiltration and macrophage activity, COX2 can suppress the immune system. Although research has demonstrated that COX2 is significantly expressed in a variety of cancers and is crucial for the formation of malignancies, the specific regulatory mechanism of COX2 in cancer cells is still unknown (6).

Inducible nitric oxide synthase (iNOS), which produces nitric oxide (NO) from L-arginine, is a key modulator of immune activation and inflammation and is present in most human illnesses. Numerous pathologies including sepsis, cancer, and neurodegeneration have been linked to diseases where iNOS is overexpressed or dysregulated (7).

The cytotoxic impact of nitric oxide is mediated via nitric oxide derivatives. Nitric oxide derivative-induced post-translational changes control the survival or demise of cancer cells. Nitric oxide derivatives act as a mediator for nitric oxide's genotoxic action. Increased levels of reactive nitrogen species, alteration in redox balance, and dysregulated redox signaling are common features of cancer progression and chemoresistance (8).

Chemokines are a class of chemoattractant regulatory proteins with a weight range of 8 to 12 kDa. By attaching to their receptors, moving leukocytes, and increasing their effects, chemokines are essential for controlling immunological responses and inflammation. Innate immune cells are

enlisted by chemokines as the initial line of defense. The impacts of other mediators (lipids, minerals, peptides, and other chemoattractants) in the inflammatory space are then integrated by these cells. The epithelial-mesenchymal transition (EMT), motility, invasion, and metastasis are all impacted by these actions. A microenvironment of chemokines generated by cancer cells and leukocyte subtypes leads to the manifestation of inflammatory cancer. Depending on the makeup/stages of cancer, the effect of chemokines can be beneficial or detrimental, homeostatic or inflammatory. Their primary duty is to ensure that immune cells are transported in a coordinated manner to the area of inflammation (9).

The CC chemokine superfamily member monocyte chemotactic protein (MCP-1) is essential for attracting and activating monocytes during angiogenesis and acute inflammation. MCP-1 is expressed by a number of cell types in the human lungs, including macrophages, endothelium, bronchial epithelial, and smooth muscle cells (10).

In the case of chronic inflammation, proinflammatory mediators, TNF-α, IL-1β, and MCP-1 can reach the lungs, which can induce inflammation in the lung and the expression of COX-2 and iNOS is up-regulated. As a result of an inflammatory stimulus, transcription of genes such as cytokines, chemokines and cyclooxygenases is activated in the nucleus. These genes may mediate lung cancer development and progression by inducing proliferation, angiogenesis, and Epithelial Mesenchymal Transition (EMT) or inhibiting apoptosis (11) (Figure1).

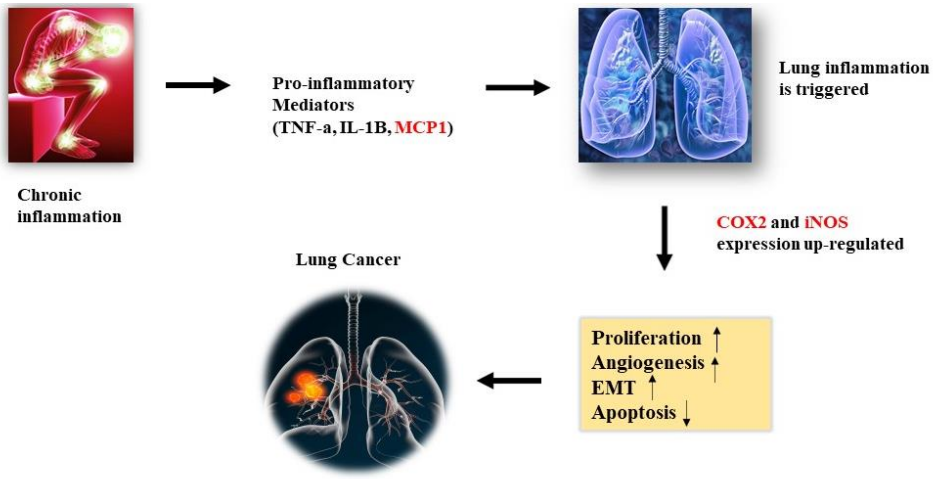

Figure1. Role of inflammatory molecules COX2, iNOS and MCP1 in lung cancer development (Figure drawn in accordance with reference (11))

In light of the information given, our study was planned to determine the relationship between lung cancer and serum levels of COX2, iNOS, and MCP-1 proteins, which are effective in the inflammatory process. In addition, these proteins have been planned with the thought that they will help in the early diagnosis and determination of therapeutic targets in lung cancer.

2. Materials and Method

2.1. Determination of study groups

This study was made after the approval of the Eskişehir Osmangazi University Clinical Research Ethics Committee with the ethics committee decision numbered 2018/12. A total of 90 patients, 36 squamous cell carcinoma, 18 small cell carcinoma, and 36 adenocarcinomas, who applied to the Eskişehir Osmangazi University Faculty of Medicine Education Practice and Research Hospital Chest Diseases outpatient clinic and agreed to participate in the study, were included in the study. For the control group,

90 healthy individuals who applied to the Eskişehir Osmangazi University Faculty of Medicine Education Practice and Research Hospital Chest Diseases outpatient clinic with various complaints and were not found to have any malignancy after the clinical examination, had no other chronic diseases and agreed to participate in the study were included. The blood taken from the patient and control groups was taken into 4 ml gel, vacuumed, disposable, endotoxin-free tubes and kept at room temperature for 2 hours, then centrifuged at 2500 rpm for 15 minutes at 4°C and the supernatant (serum) part was separated. Samples were stored at -80 °C until assayed.

2.2. Determination of serum COX2, iNOS, MCP1 and levels

Human COX2, iNOS, and MCP-1 Elisa kits (Elabscience Biotechnology Co., Ltd.) were used to determine the levels of COX2, iNOS, and MCP-1 in serum. First, samples, reagents, and standards were prepared, and then the reaction steps were carried out according to the kit protocol as follows. Finally, using a microplate reader (Thermo, MULTISKAN GO) set to 450 nm, the optical density (OD) of each well was calculated simultaneously. According to the developed standard curve, the data were computed and expressed as pg/ml or ng/ml.

2.3. Statistical evaluation of data

All analyzes were performed with the IBM SPSS Statistics 21 package program, and p-values less than 0.05 were considered substantial in all analyzes. Statistical evaluation results of protein levels of patient and control individuals are presented in Tables 1 and 2 as mean±standard deviation (M±SD).

3. Results

Serum COX2, iNOS, and MCP1 levels were evaluated between lung cancer and control groups, and a statistically substantial difference was found (p<0.001) (Table 1). In addition, COX2, iNOS, and MCP1 serum levels were found to be substantially higher in lung cancer patients compared to control individuals.

Table 1. Serum COX2, iNOS, and MCP1 Levels in Lung Cancer and Control Subjects

Group	n	COX2 (ng/ml)	Statistic	iNOS (ng/ml)	Statistic	MCP-1 (pg/mL)	Statistic
Control (M ± SD)	90	2,40 ± 1,60		189,74 ± 96,25		11,50 ± 3,04	
Lung Cancer (M ± SD)	90	6,76 ± 1,71	p<0,001	307,38 ± 126,16	p<0,001	44,71 ± 8,61	p<0,001

When COX2 serum levels were compared between lung cancer subgroups, no substantially significant difference was found (p=0.117). Likewise, iNOS and MCP1 levels were also evaluated between subgroups, and no substantial difference was detected (p=0,274, p=0,282) (Table 2).

Table 2. Comparison of serum COX2, iNOS, and MCP-1 levels among lung cancer groups

Group	Lung Cancer (M ± SD) (1) Squamous cell carcinoma (n=36)	(2) Small cell carcinoma (n=18)	(3) Adenocarcinoma (n=36)	Statistic p
COX2 (ng/ml)	6,59 ± 1,69	7,51 ± 1,71	6,56 ± 1,68	**p=0,117**
iNOS (ng/ml)	309,08 ±151,92	267,30 ± 52,31	325,25 ± 117,96	**p=0,274**
MCP-1 (pg/mL)	44,54 ±7,21	42,19 ±8,05	46,14 ± 9,97	**p=0,282**

4. Discussion

Our present understanding of immune system inflammation during carcinogenesis can be summed up as follows: Immune surveillance and the immunological sculpting of tumor heterogeneity are made possible by immunity's anti-tumorigenic role. Pro-tumorigenic inflammation, on the other hand, fosters cancer by obstructing anti-tumor immunity, modifying the tumor microenvironment to a more permissive state, and directly delivering tumor-promoting signals to cancer cells and epithelial cells (5).

The majority of human malignancies have excessive Cyclooxygenase-2 (COX2) expression, however, its specific regulation mechanism in cancer cells is yet unknown. Dai et al. In 2019, they determined the expression of COX2 and BPTF in lung cancer cell lines, mouse tumor tissues, and human samples in their study that aims to identify new regulatory factors that bind to the COX2 promoter and regulate COX2 expression and cancer cell growth. It was discovered that BPTF and COX2 expressions in lung cancer patient tumor tissues were positively correlated and that high BPTF and COX2 expressions were linked to a poor prognosis. Western blot and RT-PCR analyses examined the expression of BPTF and COX2 in NSCLC cell lines, and it was determined that BPTF and COX2 were highly expressed in both A549 and NCI-H460 lung cancer cell lines. Study results showed that BPTF cooperates with p50 (NF-κB) to regulate COX-2 expression and lung cancer growth, suggesting that the BPTF/p50/COX2 axis may be a potential therapeutic target for lung cancer (6).

Tumor-associated macrophages (TAMs) play role in several mechanisms of tumor biology including oncogenesis, drug resistance, and tumor immune escape as well as tumor metastasis. It has been determined that TAMs in osteosarcoma (OS) patients can support OS cell migration and invasion and induce epithelial-mesenchymal transition (EMT) by regulating COX-2, MMP9, and phosphorylated STAT3. In addition, the anti-metastatic effect of COX2 inhibition was determined by suppressing COX2 expression, EMT activating transcription factors, and STAT3 pathway both in vitro and in vivo. The results of the study also indicated that the COX2 inhibitor aspirin can substantially reduce the risk of lung metastases in vivo. These findings show that TAMs and COX2 may be potential targets for future anti-metastatic therapy (12).

Parallel to this knowledge, our investigation discovered that lung cancer patients had substantially higher COX2 protein levels than healthy controls. TAMs, as was previously discussed, are crucial in altering the tumor microenvironment and fostering tumor spread. Lung cancer cells have been found to respond favorably to ginsenoside Rh2 (G-Rh2), a monomeric molecule isolated from ginseng. It is still unknown, nevertheless, whether G-Rh2 can influence how TAMs differentiate and interact with the surrounding tissue. Tumor necrosis factor-alpha (TNF-a) and inducible nitric oxide synthase (iNOS) were found to be highly up-regulated in macrophages in a study looking into how G-Rh2 regulates the macrophage phenotype and affects the migration of non-small cell lung cancer (NSCLC) cells (13).

In our study, iNOS protein levels were found to be substantially higher in lung cancer patients than in control individuals.

Nitric oxide (NO) is a lipophilic, widely distributed, and transient physiological messenger that controls a number of vital physiological processes, including apoptosis, respiration, cell migration, and vasodilation. The conversion of L-arginine, NADPH, and oxygen into NO, L-citrulline, and NADP is catalyzed by all isoforms of NOS. NO can affect cells in cGMP-independent ways, such as through cGMP-induced posttranslational changes to cysteine and tyrosine residues. It also encourages mixed disulfide formation, altered protein function, altered cell cycle checkpoints, apoptosis, and

DNA repair, all of which are connected to gene transcription, genotoxic lesions, altered protein oxidation processes, and DNA repair. NO makes tumor cells more susceptible to chemotherapy agents. Hypoxia-inducible factor-1 and p53 are involved in the control of the stress response in response to the numerous effects of NO in the tumor environment, which frequently cause growth arrest, apoptosis, or adaptation in cells (14).

The potent monocyte-attracting chemokine MCP-1/CCL2 actively attracts monocytes to inflammatory and neoplastic sites. MCP-1/CCL2 can be produced by a variety of cells in the tumor microenvironment in response to various stimuli. It has been determined that microvesicles (MV) produced from lung tumor cells stimulate macrophages to release MCP-1/CCL2, which attracts monocytes that develop into macrophages that promote metastasis. These specialized macrophages foster the spread of lung cancer by producing a pre-metastatic inflammatory milieu that is essential for the survival and colonization of migrating tumorigenic cells (15).

In the tumor microenvironment, mast cells are commonly activated. Depending on the type of tumor, they can perform both pro- and anti-tumorigenic roles. The tumor microenvironment is known to contain a number of soluble factors that can regulate mast cell activation and recruitment. However, it is still unknown how mast cells are activated by tumor cells. Whether human mast cells interact with tumor-derived microvesicles (TMV) from non-small cell lung cancer (NSCLC) cells, activate them to release cytokines, and affect their capacity to migrate, was the subject of a study. According to the study, the main receptors for PKH67-labeled TMV isolated from NSCLC cell lines were mast cells. Uptake of TMV released from NSCLC cell lines or surgical lung tissue samples resulted in increased ERK phosphorylation, enhanced mast cell migration ability, and the release of cytokines and chemokines such as TNF-α and MCP-1. These data are consistent with the conclusion that TMV has the potential to affect mast cell activity and thus influence tumorigenesis through increased cytokine and chemokine release (15).

Monocyte chemotactic protein-1 (MCP-1), is a potent adipokine that is also expressed in adipose tissue and is positively linked with body obesity. Fat accumulation is a strong indicator of poor health outcomes because adipose tissue produces proinflammatory adipokines that are linked to many pathological processes, including cancer. To test the idea that MCP-1 produced by adipose tissue contributes to metastasis, male adipose MCP-1 knockdown (MCP-1 -/-) and wild-type (WT) mice were fed either the standard AIN93G diet or a high-fat diet (HFD) comprising 16% or 45% energy from soybean oil. In comparison to the equivalent controls, MCP-1 mRNA and protein levels in adipose tissue were lower after adipose MCP-1 knockdown. HFD enhanced the number of lung metastases that formed in WT mice. When WT mice were fed the AIN93G diet compared to MCP-1 -/- mice fed the HFD, the number of metastases was greater. MCP-1 -/- animals developed fewer metastases overall than WT mice, regardless of diet. Adipose MCP-1 -/- animals displayed lower plasma levels of insulin, proinflammatory adipokines, and angiogenic markers as compared to WT mice. These results suggest that adipose MCP-1 decrease may lead to the down-regulation of inflammatory and angiogenic pathways during the development of cancer. This study concluded that adipose MCP-1 deficit reduces LLC's ability to metastasize to the lungs and supports the idea that MCP-1 produced by adipose tissue aids in the spread of cancer (16).

Similarly, in our study, it was determined that MCP-1 level increased substantially in lung cancer patients compared to control individuals. However, no difference was determined in MCP1 serum levels between the groups.

5. Conclusion

Cancer biology is evolving from a "cancer cell-centered" perspective to a more comprehensive idea that assesses cancer cells with their environment, placing them in a network of stromal cells made up of

fibroblasts, vascular cells, and inflammatory immune cells that make up the tumor microenvironment.

To learn more about the mechanisms underlying potential cancer treatments and prevention measures, numerous immune system studies, including those on cancer vaccines, anti-cancer immune cells, different types of immunotherapy, anti-cancer antibodies, and biological therapies, are currently being conducted. It is now obvious that the immune system can contribute significantly to carcinogenesis at all stages. Therefore, the identification of inflammatory factors that directly or indirectly affect and support tumorigenesis and their association with cancer types is very important for the identification of new biomarkers and therapeutic targets for cancer.

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Ethics

Ethics Committee Approval: The study was approved by Eskişehir Osmangazi University Clinical Research Ethical Committee (Decision no: 12, Date:20.09.2018).

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