

Surveying the Expression of CDH1 and EGFR Genes in Patients with Anthracosis and Its Relationship with Lung Carcinoma

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Introduction: Anthracosis is known as the black lung disease and studies have shown relationships between the disease and lung carcinoma. In addition, the expression of CDH1 and EGFR genes can be used as the prognostic of anthracosis. The present study surveys the expression of CDH1 and EGFR genes in anthracosis patients and its relationship with non-small cells lung carcinoma.

Methodology: Thirty anthracosis patients diagnosed by a specialist participated in the study. The subjects were asked to sign an informed letter of consent and then, to examine the expression of CDH1 and EGFR genes through real-time PCR, tissue samples were collected. Afterward, the relationship of expression of the biomarkers with tumor staging and cancer progress was examined.

Results: Mean age of the patients was 48 ± 10.5 years. As the reference gene, 18sRNA was adopted and comparison of mean CTs at the center and edges of lesions showed no significant difference. As to CDH1 gene at the center of lesions, 12 cases out of 30 were positive, which indicates 40% sensitivity; while at the edges, 20 cases out 30 were positive. Comparison of the positive rates of CDH1 gene at the center and edges of the lesions indicated a significant difference (P -value <0.001). Moreover and with regard to EGFR gene at the center of lesions, 19 cases out 30 were positive (sensitivity = 63.3%) and five cases out of 30 were positive at the edges of lesions (control). There was also a significant difference between the positive cases of EGFR gene at the center and edges of the lesions (P -value <0.001). Clearly, the positive cases of CDH1 at the edges were higher than that at the center, while in the case of EGFR gene, positive cases at the center were higher than that at the edges. It is notable that to obtain results with higher accuracy entails increasing the sensitivity level. Following similar studies and to have higher accuracy, the experiments were carried out with three iterations.

Conclusion: In general, the results indicated a sort of relationship between anthracosis and lung carcinoma. To prove such relationship on a more solid ground, more studies with larger number of subjects are needed.

Introduction

Anthracosis is the pneumoconiosis caused by exposure to coal dust [1]. The term “anthracosis” refers to coal particles and other black pigments of which carbon is the main element [2]. Coal particles, mostly in the coalmine workers and to a less extent in city dweller, are found in the background of vocational and air pollution diseases as sediment at mucosa and submucosa; and

under bronchoscopy, they appear as black lesions with normal or deformed bronchus with high fragility [2] .

In addition to coalmine workers, the disease is found among workers in the industries related to coal [2]. In fact, occupational exposure to carbon, silicon dioxide, and quartz at work are the known causes of pulmonary anthracosis; although, there are cases without any pertinent occupation history as well [3] . In other words, anthracosis is an outcome of aggregation of carbon in the lungs after long and frequent exposure to air pollution and inhaling smock and coal dust [4]. In addition to the lungs, there are reports of anthracosis in the liver, spleen, and esophagus; the latter case should be considered as a serious disease as it demonstrates malignant melanoma [5]. In some cases, an anthracosis case is misdiagnosed as tuberculosis, lung carcinoma, or pneumonia [6].

Cytology studies on pulmonary tissues have shown that inter-bronchus anthracosis and lymphadenopathy mediastinum are concurrent with the early endocardium. Thereby, there is a probably significant relationship between anthracosis and lung carcinoma [7] .

Lung carcinoma is the most common type of cancer in men [8 9,] and more than 80% of the patients die during the first five years after diagnosis [10, 13] .

CDH1 gene encodes E-Cadherin protein [14] and it is a tumor suppressor gene with a key role in preserving cellular adhesion and stable connections in normal tissues through expression of Cadherin1 protein. In addition, the gene keeps epithelium cells in their normal layer and arrangement [15] .

The role of E-Cadherin in signaling indicates that the molecule can demonstrate different functions so that it can regulate the cell response to the external signals received by the cell. Therefore, it can regulate migration, proliferation, apoptosis, and cell differentiation [16] .

Failure of CDH1 function facilitates progress of cancer via the increase of proliferation, invasion, and metastasis [15]. The decrease of E-cadherin expression is a main molecular event effective on the failure of inter-cell adhesion system, cancer invasion, and metastasis[16] . Epidermal growth factor receptor (EGFR) receives epidermal growth factor family (EGF family) from extracellular protein ligands [17 18,] . By attaching to epidermal growth factors (EGF) and transforming growth factor (TGF), EGFR induces cellular proliferation. Activation of EGFR messaging path in cancerous cells leads to cellular proliferation, angiogenesis, metastasis, and decrease of apoptosis[19] . EGFR is highly expressed in a variety of malignancies like head and neck, colorectal, lung, breast, and bladder [20] .

The present study is an attempt to survey and compare the expression of CHD1 and EGFR genes at the center and edges of the black lesions in anthracosis patients and examine the relationship between anthracosis and lung carcinoma.

Methodology

To examine expression of CDH1 and EGFR genes, 30 patients in Masih Daneshvari Hospital-Tehran with confirmed cases of anthracosis (based on physical examinations by a specialist) who had received no treatment intervention at the time of the study were elected. After examinations by the specialist, the candidates interested in participation filled out a letter of consent. Afterward, tissue samples from the center the lesions and the unaffected tissue around the lesions (as control sample) were collected by the specialist in bronchoscopy ward.

RNA extraction

The extraction was done using Cinna pure RNA kit (cat.NO:PR891620-S). About 25-50mg fresh

tissue was placed on a plate and transferred to RNAase-DNase free micro-tubes -1.5ml. Afterward the samples were crushed to obtain a homogenized tissue and the extraction steps were implemented as per the instruction. Nano-drop device was used to evaluate quality of the extracted RNA.

cDNA preparation

Viva 2-steps RT-PCR Kit (Cat no.RTPL12) was used to extract cDNA from mRNA. The kit is featured with high sensitivity and capacity for proliferation of any type of DNA out of RNA. Afterward, specific polymers were designed in Allele ID6 and ordered for preparation.

Parameters	CDH1	EGFR	18s rRNA
F initiator	TGCCATAGATGAATTGAAGGA ATG	AAATCCTGCATGGCGCCGTG	GTAACCCGTTGAACCCCAT
Length of the primer	24	20	20
R initiator	TGTCATATATTAATTGCATAA ACACCTCA	GGTGGTTCTGGAAGTCCATC	CCATCCAATCGGTAGTAGCG
Length of the primer	29	20	20

Table 1- The specifications of the primers used in RT-PCR reaction

RT PCR reaction

CinnaGree qPCR Mix, 2X and Sina Colon Co. kit (Cat No. MM2041) were used. The kit employs EvaGreen (a fluorescence dye), which emits fluorescence light when it is attached to two-stand DNA. The light is measurable by a PCR device.

The elements of RT-PCR reaction included:

- a) Pattern sequence (2µl);
- b) Master mix (4µl);
- c) Primer based on the optimum concentration determined in the primary set up tests.
- d) Deionized distilled water to increase the final volume of the reaction to 20µl;

Cycles	Time	Temperature	Cycle step
1	10 min	95 C	Initial denaturation
	10 s	95 C	Denaturation
	60 s	65-60 C	Annealing
	20 s	72 C	Elongation

Table 2: Temperature and term of RT-PCR reaction

It is notable that reference gene was needed for internal control and 18srRNA was used in this study. After completion of each reaction, the results should be interpreted based on amplification and melt curves. The collected data was analyzed in SPSS (V20) and significance of the results was determined using t-test.

Results

The study was carried out on 30 patients including six women and 24 men. Mean age of the subjects was 48 ± 10.5 years. 18srRNA gene was used as the reference gene and comparison of mean "ct" values for the center and edges of the lesions indicated no significant difference (p-value=0.263), which made the gene suitable as reference gene.

As to CDH1 gene, there was 12 positive cases out of 30 at the center of lesions (sensitivity = 40%) and 20 positive cases out of 30 at the edges. Comparison of positive CDH1 gene cases at the center and edges of lesions indicated significant differences (P-value<0.001) (Fig.1). With regard to EGFR gene, there were 19 positive cases out of 30 at the center of lesions (sensitivity = 63.3 %) and five positive cases out of 30 at the edges of lesions (control). There was a significant difference between the center and edges of lesions in terms of positive cases of EGFR (p-value<0.001).

Figure 1: Evaluation of CDH1, EGFR mRNA expression in the center and edges of the lesions by RT PCR.

Clearly, the rate of positive CDH1 gene at the edges of lesions was higher than that at the center, while the rate of positive EGFR gene at the center of lesions was higher than that at the edges. In light of this, it must be taken into account that the patients are at high risk of NSCLC, which can be a good guideline for treatments and follow ups. It is notable that to obtain better results and higher sensitivity, following similar studies, the experiments were carried out with three iterations.

Difference in expression of CDH1 and EGFR genes at the center and edges of lesions

Relative level of difference between the markers was measured for the two groups. This was done through $\Delta\Delta ct$ for CDH1 and EGFR genes [21]. Value of $\Delta\Delta ct$ for CDH1 biomarkers was -1.8 and calculating 2 to the power of $-\Delta\Delta ct$ yields the difference between the markers expressions. The number of primary copies of the markers at the edges of lesions was on average 3.4 times higher than that at the center of lesions. Value of $\Delta\Delta ct$ for EGFR was obtained equal to -1.5 and calculating 2 to the power of $-\Delta\Delta ct$ yields the difference between the markers expressions. The number of the primary copies of the markers at the center of lesions was 2.8 times higher than that of the edges of lesions (Fig. 2)

Figure 2: Difference in expression of CDH1 and EGFR genes at the center and edges of lesions. :

Discussion

Anthraxosis or the black disease is the outcome of aggregation of carbon pigments in the bronches, which causes changes in and damages to the bronches that appear as black lesions. These black lesions have been rarely studied by other studies [2] .

Although, in the industrial countries have managed to decrease the prevalence rate of the disease, it is still highly prevalent in undeveloped countries [22] .

Taking into account the growing rate anthracosis in the developing countries and the large number of deaths due to the disease in these countries and given the confirmed relationship between the disease and clinical specifications of different types of lung cancer [4] , early diagnosis of the disease of great importance.

The relationship between the black lesions of anthracosis and NSCLC was examined. To this end, the expression of CDH1 and EGFR genes that change in the cancerous conditions was examined [14 19,] .

CDH1 molecule (E-cadherin) is an adhesive molecule that plays a role in metastasis and tumor invasion. It is a calcium dependent molecule that causes inter-cellular adhesion [16]. In most of the cases, epithelial cancerous cells completely or relatively lose their E-cadherin molecule when they start transformation to malignant conditions [16]. This is the case in lung carcinoma and changes in methylation of DNA and histone acetylation along with other chromatin related protein changes can induce epigenetic changes and affect expression of genes. Therefore, the disruption of gene expression has to do with the cancer [23,24].

By attaching to EGF ligands and TGF, EGFR induces cellular proliferation. Activation of EGFR signaling path in cancerous cells leads to cellular proliferation, angiogenesis, metastasis, and decrease in apoptosis [19].

Shoji et al. (2006) studied 224 subjects with NSCLC to examine methylation and expression of CDH1 and P16. In general, the improper methylation of promoter of CPG islands is a major deactivation mechanism of tumor suppressor and tumor dependent genes [25]. They also utilized RT-PCR method for diagnosing and found that the expression of the protein was deactivated through methylation. Thereby, the expression of CDH1 and P16 genes and methylation analysis are highly helpful in the clinical diagnosis of NSCLC. CDH1 gene can be used to diagnose NSCLC.

Lia Yi et al. (2002) studied 42 patients with follicular thyroid cancer and 54 patients with papillary thyroid cancer in terms of E-cadherin expression. Their results showed that the expression of E-cadherin in metastatic papillary cancer was higher than that of the cases without metastasis [26]. To determine the expression of CDH1 they used immunohistochemistry and, consistent with our findings, reported that the positive expression of CDH1 in cancerous tissue decreased with metastasis of lymph glands. Thereby, there is a significant relationship between expression E-cadherin and metastasis to lymph glands. Yango et al. (2012) reported a significant relationship between the cancer and the over expression of EGFR, depth of invasion, and metastasis of lymph glands. They also reported over expression and high copy number of EGFR gene were commonly observed in NSCLC cases [27]. They used fluorescent in situ hybridization (FISH) to determine EGFR expression. In the present study, the increase of expression EGFR at the center of lesions indicated the oncogenic role of EGFR in NSCLC. Generally, EGFR can be used as suitable target in cancer treatment.

In Conclusion, Based on the findings, CDH1 and EGFR biomarkers increased in the black lesions of lung tissue, while the level of EGFR biomarkers in the patient group (center of lesions) was notably higher than that of the control group (normal tissue at the edges of lesions). Moreover, the level of CDH1 biomarkers in the patients group (center of lesions) was notably higher than that of the control group (normal tissue at the edges of lesions). Thereby, anthracosis patients are at high risk of NSCLC, which is a valuable guideline for treatment and follow up services to the patients.

To have results with higher reliability, future studies need to focus on larger sample groups. In addition, examining other biomarkers in lung cancer can improve accuracy of screening test for this type of cancer.

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