Sialic Acids of Saliva in Primary and Differential Diagnosis of Lung Cancer

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The aim of the study was to explore the option of using sialic acid of saliva as a marker for primary and differential diagnosis of lung cancer.

Materials and Methods. The study included 1903 subjects divided as follows: the main group (lung cancer, n=337, and non-malignant lung diseases, n=108), the comparison group (other types of oncological diseases, n=1033), and the control group (generally healthy, n=425). All participants filled the medical questionnaires and presented the histological verification of their diagnoses; then they underwent biochemical examination of their saliva samples. The level of sialic acids and the content of mucin in the saliva were determined spectrophotometrically.

Results. We found that the average level of sialic acids in the control group (0.270±0.037 mmol/L) was significantly higher than that in lung cancer (0.138±0.006 mmol/L) or non-tumor diseases of the lungs (0.148±0.003 mmol/L). The saliva content of sialic acids did not significantly differ between various histological types of lung cancer (0.175±0.027 and 0.166±0.024 mmol/L for squamous cell lung cancer and adenocarcinoma, respectively). We also noted that in patients with metastatic lung cancer, the level of sialic acids in the saliva was the lowest as compared with the generally healthy subjects.

Conclusion. The level of sialic acids in the saliva decreases both in patients with lung cancer and in patients with non-malignant lung diseases. These results rationalize the option of using this parameter for the primary diagnosis of lung disorders as a whole; however, for the differential diagnosis of various lung diseases, the level of saliva sialic acids is of little value.

Key words: diagnosis of lung cancer; biochemistry of saliva; sialic acids; mucin; clinical laboratory diagnostics.

Introduction

Lung cancer (LC) is the most common malignant tumor and one of the main causes of death in cancer patients [1, 2]. Such methods as chest X-ray and sputum cytology were found ineffective in the diagnosis of LC [3]. At present, the low-dose chest CT is recommended for this purpose, but its use is limited to the 55–74 year olds, heavy smokers or those who quit smoking less than 15 years ago. High hopes are placed on the diagnostic value of the early molecular markers of LC (CEA, Cyfra 21-1, CA 72-4 for adenocarcinoma; Cyfra 21-1, SCC, CEA for squamous and large cell LC; ProGRP, HCE, CEA for small cell LC) [4, 5]. However, their use is often limited to clarifying the diagnosis, evaluating the treatment efficacy, predicting the prognosis, or early detecting the cancer recurrence; only in a few cases, these markers have been used for making the primary diagnosis of cancer. In recent years, the possibility of using the known and new tumor markers in the primary and differential diagnosis of LC has been widely studied. At the end of the last century, the idea of using sialic acids for diagnostic purposes had been proposed [6]. A number of studies demonstrated the feasibility of determining sialic acids for the diagnosis of colorectal cancer [7], thyroid cancer [8], prostate cancer [9], ovary cancer [10], and oral cavity cancer [11]. However, the diagnostic value of sialic acids as a marker of LC was never explored. It should be mentioned that in the above studies, the level of sialic acids was measured in the blood or tissues, whereas other authors suggested that the use of saliva might be more informative [12, 13].

Sialic (neuraminic) acids are multifunctional molecules present in all tissues and fluids of the human body; the highest amounts are found in the human saliva [12, 13]. Normally, sialic acids are not present in the free form but are parts of various carbohydrate-containing macromolecules, such as glycoproteins (i.e. mucins of the saliva), glycolipids, and oligosaccharides [14, 15]. Glycoproteins are complex proteins containing up to 80% of carbohydrates, namely: N-acetylglicosamine, N-acetylgalactosamine, galactose, fucose, mannose,
Sialic acids are present on receptors of cell membranes and are capable of masking cancer cells from recognition by the immune system [16]. There is abundant evidence for a statistical relation between the number of sialic acid residues in the membrane glycoproteins and carcinogenesis [14, 17, 18].

The aim of the study was to explore the option of using sialic acid of saliva as a marker for primary and differential diagnosis of lung cancer.

Materials and Methods

This case-control study included 1903 participants separated into three groups: the main group (with lung diseases), the comparison group (with cancer other than LC), and the control group (generally healthy). The subjects were enrolled simultaneously into all three groups. All participants underwent biochemical testing of their saliva samples.

The main group included 445 patients with pulmonary diseases, including 337 patients with histologically confirmed LC (98 females, 239 males) and 108 patients with non-malignant pulmonary diseases (7 with chronic pneumonia, 20 with tuberculosis, 28 with hamartoma, 10 with sarcoidosis, 24 with fibrosis, and others). The group of patients with LC was further divided into subgroups according to the following characteristics: sex and age, histological type of tumor (adenocarcinoma, squamous cell carcinoma), stage of the disease according to the TNM Classification of Malignant Tumours.

The comparison group included 1033 patients with other types of cancer and consisted of two subgroups. The first one included 302 male patients (129 with prostate cancer, 150 with malignant neoplasms of the gastrointestinal tract, 59 with breast cancer, 118 with gastrointestinal cancer, 225 with non-malignant pulmonary diseases, 73 patients with sarcoidosis, 24 with fibrosis, and others). The other subgroup consisted of 731 female patients (400 with uterine, cervical, and ovarian cancers, 180 with breast cancer, 118 with gastrointestinal cancer, and 33 with malignant neoplasms of the genitourinary system).

The control group included 425 generally healthy patients (192 males and 233 females) who had neither pulmonary nor oncological diseases according to the most recent clinical checkup.

The groups of subjects were formed in agreement with the rules for conducting clinical trials after obtaining an informed consent.

The following inclusion criteria applied: age 30–75 years; no treatment at the time of the study (including but not limited to surgery, chemotherapy or radiation); no signs of active infection (including purulent processes); consent for oral cavity sanitation.

The candidates who had no histological verification of his/her diagnosis were excluded.

The study was conducted in accordance with the Helsinki Declaration (2013) after the approval of the Ethics Committee of the Clinical Oncology Hospital.

Saliva samples of 1 ml were taken from all patients before the treatment commenced. The determination of sialic acids included the hydrolysis of protein-free filtrates; at this step, sialic acids were released from sialoglycoproteins. Next, the reaction mixture was added with acetic and sulfuric acids and placed in a boiling water bath; the resulting product is a colored substance that reflects the amount of sialic acids in the sample [19]. One ml of trichloroacetic acid, 2 ml of distilled water, and 0.6 ml of saliva were placed into test tubes. After thorough mixing the tubes were put in a boiling water bath for 5 min, then cooled, and centrifuged at 2000 rpm to separate the precipitate. Then 0.4 ml of acetic-sulfuric acid mixture was added to the supernatant and the samples were reheated in a boiling water bath for 15 min, cooled and added with 2 ml of distilled water. After that, the optical density was measured using a green filter (500–560 nm) in a cuvette of 10 mm thick.

The amount of mucin in the saliva was determined spectrophotometrically by the difference in the protein concentration between the starting material and the supernatant formed after the acidic precipitation of mucin. To that end, two test samples were prepared: the first contained the saliva and the working reagent; the second the supernatant and the working reagent. The standard sample contained an aqueous solution of albumin at 0.25 g/L and the working reagent. The control sample containing distilled water and the working reagent was prepared by mixing a solution of bromphenol blue at 1.2 g/L and a buffered solution (pH 3.0) containing 320 mmol/L citric acid and 160 mmol/L sodium phosphate at the ratio of 2:23. The contents of each sample were mixed and incubated for 10 min. The optical densities of the test and standard samples were determined at a wavelength of 620 nm and related to the control sample.

The statistical analysis of the obtained data was performed using the Statistica 10.0 (StatSoft) and package R (version 3.2.3) software. The graphs representing the experimental results were developed using the ggplot2 (version 2.0.0) package.

Results and Discussion

Sialic acids in the normal saliva. At the initial stage of the study, the concentration of sialic acids in the normal saliva was determined. It was found that for women (mean age 45.89±1.59 years) the normal concentration of sialic acids was 0.244±0.023 mmol/L, for men (mean age 41.86±1.53 years) — 0.285±0.025 mmol/L, which agrees well with the published data, despite the differences in the technique [20].

It was also found that the level of sialic acids in healthy individuals slightly increased with age, but no statistically significant sex or age-related differences
Table 1
Concentrations of sialic acids (mmol/L) in the saliva of generally healthy subjects (control)

| Age (years) | Females (n=233) | Males (n=192) |
|-------------|-----------------|---------------|
| 40–49       | 0.226±0.039     | 0.300±0.033   |
| 50–59       | 0.237±0.040     | 0.243±0.041   |
| 60–69       | 0.246±0.077     | 0.258±0.056   |
| Over 70     | 0.265±0.083     | 0.260±0.076   |

Figure 1. Distribution density of sialic acid concentrations in groups of generally healthy male subjects (HEALTHY), male patients with malignant neoplasms of the lung (MN_LUNG), the gastrointestinal tract (MN_GIT), the genitourinary system (MN_GS), or the prostate (MN_PROSTATA).

Figure 2. Distribution density of sialic acid concentrations in groups of generally healthy female subjects (HEALTHY), female patients with malignant neoplasms of the lung (MN_LUNG), and patients with malignant neoplasms of the uterus, cervix, and ovaries (MN_GYN).

were noted (Table 1). The results allowed us to use the mean values for the reference in further calculations.

*Sialic acids of the saliva in different types of cancer.* To test the potential use of the sialic acid level in the diagnosis of cancer, two gender-specific studies were conducted. Firstly, the distribution patterns of sialic acid concentrations were determined in 624 male subjects, including 192 generally healthy individuals, 130 patients with LC, 129 patients with malignant neoplasms of the gastrointestinal tract, 59 with malignant neoplasms of the genitourinary system, and 114 with prostate cancer (Figure 1).

It was found that the average levels of sialic acids in the control group (0.285±0.025 mmol/L) and in patients with malignant neoplasms of the gastrointestinal tract, the genitourinary system or the prostate were close (0.231±0.021, 0.231±0.049, and 0.236±0.009 mmol/L, respectively), whereas in LC patients this value was notably lower (0.138±0.006 mmol/L).

In a second study, 1014 female patients were examined: of those, 233 generally healthy, 400 with malignant neoplasms of the uterus, cervix, and ovaries, 180 with breast cancer, 118 with gastrointestinal cancer, 50 with LC, and 33 with malignant neoplasms of the genitourinary system. The average concentration of sialic acids in the control group was 0.244±0.023 mmol/L. A significantly lower level of sialic acids in patients with LC (0.148±0.003), as well as in patients with uterine/cervical/ovarian malignant neoplasms (0.169±0.003 mmol/L) was found (Figure 2). In all other groups, there were no significant differences between the cancer patients and the generally healthy subjects.

Based on these results, we proposed that patients with LC had a significantly different level of sialic acids as compared with all other groups included in the study. To test this hypothesis, we evaluated the distribution and homogeneity of the dispersions within the groups. According to the Shapiro–Wilk criterion, the distribution of the sialic acid levels did not obey the normal distribution pattern (p<0.05). The test for homogeneity of dispersions in the groups (the Bartlett test) showed that the dispersions were not homogeneous within the groups (p=0.00017). Therefore, we later turned to non-parametric statistical methods to compare the obtained results. The Wilcoxon and Mann–Whitney tests for paired groups showed that the distribution of the sialic acid concentration data in LC patients differed from both the control group and groups with other oncological diseases (p=0.0000). There were no differences in the data distribution between the groups with other types of cancer (p>0.05). Therefore, the distribution density...
curve for the LC group is located closer to the Y axis than that for the other groups.

The obtained results confirm the reports claiming that the determination of the level of sialic acids can be used for the diagnosis of LC and ovarian cancer [10, 21]. In addition, our studies have shown that this diagnostic approach can be relevant to malignant neoplasms of the body and cervix of the uterus.

**Sialic acids and differential diagnosis of lung diseases.** At the next stage, we compared the levels of saliva sialic acids in LC vs non-malignant pulmonary diseases. We found abnormally low levels of sialic acids in both groups of patients: 0.138±0.006 vs 0.148±0.003 mmol/L (Figure 3).

Specifically, for hamartoma, tuberculoma, and inflammatory lung diseases, the average content of sialic acids was 0.154±0.035, 0.163±0.054, and 0.098±0.030 mmol/L, respectively. Apparently, the presence of an active inflammatory process in the lungs decreases the level of sialic acids to values lower than those in LC; that factor can lead to a false positive result in making the LC diagnosis and, therefore, should be considered as a limitation of the proposed method. Among non-malignant pulmonary diseases, the level of sialic acids in sarcoidosis (0.201±0.041 mmol/L) seems like an isolated case, probably, due to the formation of noncaseating granulomas in the lungs [22, 23]. Thus, the decrease in the level of sialic acids observed both in LC and non-malignant pulmonary diseases, confirms the possibility of using this parameter for the diagnosis of lung diseases as a whole, but its use for the differential diagnosis of pulmonary diseases is yet to be proved.

**Sialic acids in different types of lung cancer.** The overwhelming majority of LC cases are represented by adenocarcinoma and squamous cell carcinoma; together they comprise about 85% of total LC cases. In this study, the level of saliva sialic acids in different histological types of LC differed insignificantly: 0.189±0.066 mmol/L for small cell LC, 0.175±0.027 mmol/L for squamous cell LC, and 0.166±0.024 mmol/L for adenocarcinoma. These results, therefore, rationalize the use of the level of sialic acids for the diagnosis of the most common histological types of LC irrespective to the size and the nature of the tumor (Table 2). However, the diagnostic value of this parameter falls short of discerning between different histological types of LC.

The obtained data show that in patients with metastatic LC, the level of sialic acids is the lowest as compared with the generally healthy individuals (see Tables 1, 2).

Sex and age of patients with LC, according to our data, have no sizable effects on the concentration of sialic acids in the saliva and thus do not diminish its diagnostic significance (Table 3).

Thus, the level of saliva sialic acids decreases both in LC and in non-tumor pulmonary diseases. This result provides support to the potential use of this marker for primary diagnosis of lung pathology as a whole.

### Table 2

| Stage of lung cancer | Squamous cell lung cancer | Adenocarcinoma |
|----------------------|--------------------------|----------------|
| T<sub>1</sub>        | No data                  | 0.155±0.041    |
| T<sub>2</sub>        | 0.162±0.027              | 0.188±0.043    |
| T<sub>3</sub>        | 0.147±0.040              | 0.118±0.026    |
| T<sub>4</sub> no metastases | 0.164±0.041            | 0.174±0.089    |
| T<sub>4</sub> with metastases | 0.155±0.031          | 0.143±0.034    |

### Table 3

| Age (years) | Females       | Males        |
|-------------|---------------|--------------|
| 40–49       | 0.141±0.049   | 0.129±0.037  |
| 50–59       | 0.116±0.031   | 0.132±0.031  |
| 60–69       | 0.101±0.029   | 0.156±0.040  |
| Over 70     | 0.137±0.046   | 0.133±0.064  |

![Figure 3. Distribution density of sialic acid concentrations in groups of generally healthy subjects (HEALTHY), patients with malignant neoplasms of the lung (MN_LUNG), and patients with non-malignant diseases of the lung (NMD_LUNG)](image-url)
However, the level of sialic acids is of little value in the cases where the differential diagnosis of lung diseases is needed.

**Discussion on the dynamics of sialic acids in the saliva.** There are conflicting reports on the blood content of sialic acids in patients with LC. According to some authors [6], this level is significantly higher than those in healthy donors or in patients with non-tumor lung diseases. In other studies [24], no significant differences in the level of sialic acids in the blood and the bronchial lavage fluid between patients with LC and those with non-tumor lung diseases were found. The authors [25] note that the increase in the level of sialic acids in the blood of LC patients positively correlates with the degree of metastatic spread of this tumor.

It should be noted that the level of sialic acids relates to the level of acute phase proteins, in particular α-1-acid glycoprotein, whose concentration would increase in any pathological process [16]. Most of the α-1-acid glycoprotein molecule is represented by a carbohydrate component characterized by the presence of terminal N-acetyllneuraminic acid residues, i.e. sialic acids. The high content of sialic residues in the carbohydrate chains helps masking the glycan antigenic determinants on cancer cells [17]. A decrease in the number of terminal N-acetyllneuraminic acid residues causes a release of free sialic acids into the blood. In the blood of healthy individuals, free sialic acids are found in small amounts [26]. The total level of detectable sialic acids is the sum of two fractions: the glycoconjugate residues and the free sialic acids; it is, therefore, reflects the content of sialic acids (free + bound) in the given biological sample [15]. A negative correlation between the concentration of sialic acids and the amount of α-1-acid glycoprotein was reported in patients with myeloproliferative diseases but not in healthy individuals [15]. It is proposed that the imbalanced glycosylation of cancer cells, in particular the increased level of sialic residues in cell membranes, is associated with the developing malignancy and with the invasive and metastatic potential [27]. It was also found that the use of certain inhibitors of sialic acid metabolism could reduce the malignant potential of cancer cells. Removal of sialic residues from the tumor cell membranes reduces the cell growth and makes those cells more vulnerable to the immune attack.

However, contrary to the blood with its increased content of sialic acids in neoplastic processes, the saliva shows the opposite trend, namely a reduction in the content of sialic acids. We suggested this might be due to the specific nature of this biological fluid, in particular, the high content of mucin.

To test this hypothesis, we determined the content of saliva-associated mucin in patients participating in this study. We have found that the saliva of generally healthy patients contains more mucin as compared to patients with LC (Table 4), with a weak positive correlation between the levels of mucin and sialic acids in the saliva samples (R=0.34, p=0.000).

In all likelihood, the saliva of healthy people is dominated by sialomucins, whereas in patients with lung diseases, neutral and acidic mucins prevail [28]. In these developments, the tumor cells actively bind sialic acids [29], which may explain why the level of free sialic acid in the saliva of patients with LC is below normal.

## Conclusion

In patients with lung cancer or non-malignant pulmonary diseases, the level of sialic acids in the saliva is abnormally low. This parameter can, therefore, be used for the primary diagnosis of pulmonary diseases in general; for the differential diagnosis of lung disorders, the level of sialic acid is, however, uninformative.

Among the oncological diseases included in the study, the level of saliva sialic acids has a potential diagnostic value for patients with lung, ovarian, uterine, and cervical cancers.

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### Table 4

| Gender- and age-associated differences in the saliva mucin content (mg/L) among patients with lung cancer and generally healthy subjects |
|---------------------------------------------------------------|
| **Age (years)** | **Females** | **Males** |
| General | Lung cancer | Generally healthy | Lung cancer |
|---|---|---|---|
| Generally healthy | Lung cancer | Generally healthy | Lung cancer |
| 40–49 | 1.15±0.18 | 0.87±0.41 | 1.19±0.12 | 0.99±0.34 |
| 50–59 | 1.17±0.16 | 0.77±0.23 | 1.23±0.19 | 0.81±0.27 |
| 60–69 | 1.18±0.33 | 1.06±0.29 | 1.48±0.49 | 0.80±0.13 |
| Over 70 | 1.71±0.60 | 0.97±0.35 | 1.21±0.38 | 0.83±0.40 |

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