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Diagnostic Tool for Non-Small Cell Lung Cancer (NSCLC) Lyophilized Serum †

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Abstract: Background and Objectives: The international protocol used to diagnosis Non-Small Cell Lung Cancer (NSCLC) usually faced an inappropriate result due to the poor diagnostic ability in early stage. Carcinoembryonic Antigen (CEA), an established serum tumor markers that is using for NSCLC diagnosis, has a limited sensitivity and specificity, but still the predominant complementary tool detection in where its results is confirmed the diagnostic radiology finding (PET-CT). Unfortunately, the limited range of its sensitivity is unable to classify approximately one third of patients suffering from NSCLC. Due to a huge number of patients is lately classified as NSCLC; the efficacy of the offered treatment is limited. Hence, the importance of discover, improve, and establish a new technique that participates in the NSCLC diagnosis indeed urgent. **Methods:** The low angle x-ray scattering (LAXS) technique was applied on the lyophilized serum of NSCLC patients to create patient profile that able to distinguish the molecular difference between NSCLC patients avoiding the undesirable radiation exposure to the patients. **Results:** The created LAXS profile is characterized by two peaks. The first scattering peak at 4.8° is sensitive to molecular alteration in protein structure that is the main characteristic difference from normal serum. Comparing the measurements of LAXS profiles of NSCLC with normal sera; the unique first scattering peak at 4.8° is elucidated a characterization shape and profile for NSCLC and normal individuals. **Conclusion:** Using LAXS technique gives us full details at molecular level that is introducing as a promising tool which could be a supporter in NSCLC early detection.

Keywords: Non-Small Cell Lung Cancer (NSCLC); low-angle X-ray scattering (LAXS) technique; lyophilized serum

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1. Introduction:

Cancer is classified as the second disease causing mortality after cardiovascular disease wherever in developing or developed countries [1]. Lung cancer is leading the mortality among cancer patient in the United States [1], China [2] and globally. It is divided into two main subtypes according to its histologic classification; small cell lung cancer (SCLC) its ratio is 15-20% [2], while Non-small cell lung cancer (NSCLC) is distributed among lung cancer patient ≈ 80-85% worldwide [1]. According to the National Comprehensive Cancer Network (NCCN) clinical guideline 2017; less than one fifth of lung cancer patients (17.7%) are alive over five years after diagnosis [1].

NSCLC patients usually develop late stage before being diagnosed, as a result of; the initial diagnosis is based on clinical signs, and bio-sample laboratory investigations; the diagnosis is confirmed with molecular diagnostic imaging (i.e. PET- CT, fMRI) [2, 3].

In comparison, the importance of the histologic examination and its classification is arising from its appropriate sensitivity to the treatment regimen [2].

The patients suffering from this type of cancer mainly are diagnosed by tumor markers whether histologic biopsy or serologic and confirmed with radiologic investigations (PET-CT) [4]. High-risk group of lung cancer includes smokers. The CT routinely screening enhanced the surveillance rate of 20% of the high-risk group [3]. The main obstacle to doing CT screening for each patient is the undesirable radiation exposure hazard, while the histologic biopsy is difficult to manipulate more than one time [4]. Several biomarkers have emerged as predictive and prognostic markers for NSCLC. The biomarkers involved in NSCLC prediction include the fusion of ALK with oncogene (e.g. echinoderm microtubule-associated protein-like 4), rearrangements of ROS1 gene, and inductive sensitization of EGFR mutations [5, 6]. The NSCLC patients suffering from sensitizing EGFR mutations predominantly will be chemo-resistant to following therapeutics agents; erlotinib, gefitinib, or afatinib in about nine to thirteen months of EGFR TKI therapy [1].

Two decades ago, Low-angle x-ray scattering (LAXS) technique has previously shown an applicable promising biophysical tool that has clinical sensitivity towards the structural alterations in lyophilized human serum [7]. Moreover, a scattering peak, which produces in this technique, was elucidated more specific information to the induced molecular alteration in the structural serum proteins level [7,8, 9]. Moreover, previous studies elucidated the sensitivity of LAXS technique to monitor any changing at the molecular level structure in biological samples [7] to distinguish characteristic profiles for normal and neoplastic breast tissues and the improvement of enhanced imaging techniques [9]. Moreover, it was used in the discriminate criteria of tissues [10].

Clinically, three tumor markers; carcinoembryonic antigen (CEA), cytokeratin 19 fragments antigen (CYFRA21-1), and squamous cell carcinoma antigen (SCCAg), are approved to diagnostic process of NSCLC with combination of the histopathologic examinations and radiologic investigations PET-CT [10]. In spite of the histopathology is the golden marker for NSCLC diagnosis; the manipulation of patient specimen biopsy or surgical specimen is reducing its accuracy [10].

2. Materials and Methods:

2.1. Collection and preparation of patient's samples

The regulations and rules of the Egyptian National Cancer Institute (NCI) Cairo University are governing this study during a period of one year. The ethical approval including -patient consent- for this study isn't necessary , due to the patient samples and their data were taken from the routine clinical investigations from the departments of clinical pathology and diagnostic radiology as anonymous samples. After that, their clinical data results and residual samples were collected as anonymous to protect the patient rights.

Blood samples were collected from 50 samples; ten samples were healthy individuals (5 males and 5 females), 10 samples were diagnosed as high risk group patients (seven males and three females) and 30 as NSCLC patients (25 males and five females). The NCI clinicians diagnosed NSCLC patients according to the National comprehensive Cancer Network (NCCN) version 5, 2017[1]; routinely clinical investigations; tumor marker and diagnostic radiology were done, and collect the results to compare with the LAXS technique. Internationally the NSCLC distribution according to sex is different from Egypt; where the female is not predominant, that is due to the high-risk group mainly in men (smokers). The distribution of the age of the collected samples for the three groups is lightly matched. No patient suffering from NSCLC below 40 years of age and it starts to increase for samples of patients above 40 years up to 69 years of age. The whole clinical data for each group is illustrated in table 1.

The thirty NSCLC patients were classified according to their tumor grading; 8 patients (grade I), 17 patients (grade II) and 5 patients (grade III) while no one grade IV NSCLC was included in this study. The patients samples' in this study were collected via venipuncture process were clotted in 37 °C temperature for half hour, after that, the

blood samples were centrifuged for ten minutes at 5040 xg (3000 rpm). Then, the supernatant patients' sera were separately collected and kept at −80°C; according to the previous research which was elucidated the suitable temperature for storing biological sample for long period is −80°C; this condition does not affect the characteristic scattering behavior [7]. The collected samples were lyophilized by using a freeze dryer (Edwards, UK) at minus fifty Celsius and negative vacuum pressure of 6.4 mbar in magnitude for six hours to complete water removal from the samples and then were kept in dry sealed plastic tubes at at minus eighty Celsius. For LAXS measurements, the samples have to warm up in the room temperature.

Table 1. clinical data of investigated groups.

	Control	**High Risk Group (Smokers)**	**NSCLC Group**
	(n = 10)	(n = 10)	(n = 30)
Mean age (years)	42 ± 3.5	48 ± 3.1	50 ± 9
Sex:			
M	5	7	25
F	5	3	5
UICC stage:			
I	-----	-----	8
II	------	-----	17
III	------	------	5

2.2. Measurements of X-ray scattering

The lyophilized powdered serum was smeared on glass mounted vertically in the rotating holder of the Shimadzu x-ray diffractometer to investigate the scattering profile for each individual. The operating condition of this instrument was working in reflection geometry at 40kV and 30 mA, using a copper target to produce 8.047 keV mainly high collimated x-rays beam. The scattering angles were investigated in this study from 2° up to 30° with steps of 0.25°. The rotation was in (θ–2θ) mode. Sodium iodide crystal with graphite monochromator in scintillation detector was collecting the scattering data and interfacing to the computer.

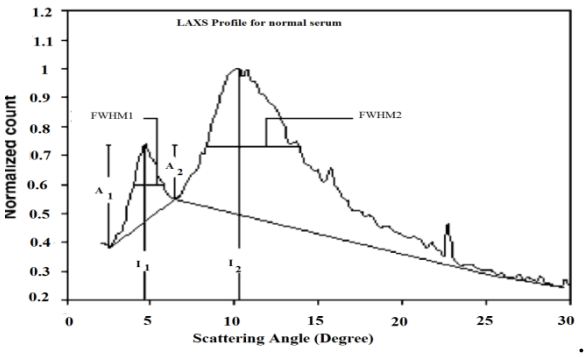

Figure 1. The Calculation of the characterized parameters measured from the scattering profile of normal serum.

2.3. Calculation and analysis the parameters from LAXS data profile:

Table 2 presents the characterized parameters that were calculated from LAXS profiles of patients sera. Figure 1 elucidated the calculation processes of these parameters. First parameter is the full width at half maximum for the first and second peaks which were acronymic known as; $FWHM_1$ and $FWHM_2$, they have the scattering degrees 4.8° and 10.5°, respectively.

To estimate FWHM; plotting the base line for each peak which were making a reference to calculate their values as illustrated in figure 1. The percentage ratio of the first and second scattering peaks values (I_1/I_2%) were inserted in table 2. Moreover, the amplitudes of the rising and falling edges of peaks 1 and 2 were tabulated as represent their values A1 and A_2 respectively.

Table 2. Mean values of the measured parameters for low-angle x-ray scattering scanned data from normal, high risk group and NSCLC serum samples.

	Normal Serum	**High Risk Group**	**NSCLC**		
	(n = 10)	**(n = 10)**	**(n = 30)**	***F*-Ratio**	***p*-Value**
$FWHM_1$ (deg)	1.96 ± 0.12 [a]	1.95± 0.2 [a]	2.19 ± 0.20 [b]	4.906	0.0120
$FWHM_2$ (deg)	5.23 ± 0.14	5.24 ± 0.22	5.39 ± 0.26	1.898	0.1600 [c]
Peak position 1 (deg)	4.78± 0.15 [a]	4.93 ± 0.22 [b]	5.08 ± 0.16 [b]	8.515	0.0005
Peak position 2 (deg)	10.52 ± 0.16 [a,b]	10.45 ± 0.22 [a.]	10.62 ± 0.13 [b]	3.878	0.0270
I_1/I_2%	55.14 ± 2.32[a]	54.20 ± 1.42 [a,b]	53.10 ± 1.74 [b]	3.324	0.0440
A_2/A_1%	45.64 ± 5.80 [a]	38.20 ± 3.82 [b]	33.80 ± 3.81 [b]	11.411	0.0001
Counts under peak 1	7.12 ± 0.22 [a]	6.99 ± 0.16 [a]	6..62 ± 0.19 [b]	20.112	0.0001

[a] Statistically classified group a. [b] Statistically classified group b which is significantly different to group a. [c] NS: non-significant.

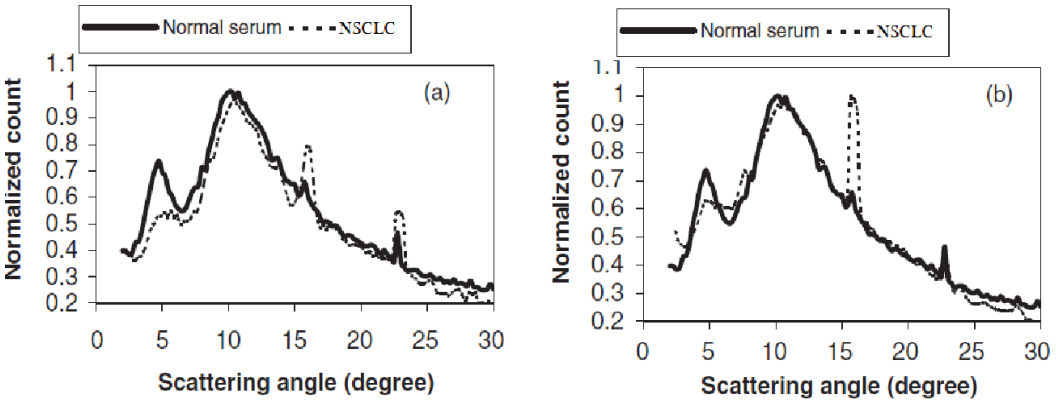

Figure 2. LAXS profiles of NSCLC compared to normal individual.

The final manipulated procedure for the characterized measured parameters was statistically analyzed by using the Statistical Package for the Social Sciences (SPSS) version 24. The representing forms of our data were tabulated as mean ± standard error of the different parameters (table 2). Analysis of variance (ANOVA) test was compared the mean values of each characterized parameters. Individual values for each parameter was used to count its mean, when the compared groups is statistically significant, an additional test was followed by Duncan's multiple range test the discrimination of investigated groups.

According to the world health organization (WHO) classification, NSCLC is distributed more than 80% among lung cancer patients [1]; moreover, the latent diagnosis of NSCLS is mainly a medical problem due to the advanced cancer stage. While the routinely medical investigation is dependent on general signs and symptoms beside laboratory markers, resulted from this finds are diagnostically supported by radiologic imaging (PET/CT scan, brain MRI).

3. Results and Discussion:

The LAXS characterized parameters are shown in figure 1 for normal sample. High risk group and NSCLC samples are presented in table 2. To be easy comparable and applicable, all graphs are normalized to unity at the second peak of scattering at 10.5° and a maximum of three-point average is plotted for each graph. Figure 2 elucidates the present of two relatively broad scattering peaks which were analyzed in this study, while a number of sharp diffraction peaks are presented due to the NaCl crystals in serum sample [8]; those sharp peaks amplitudes are differed from individual samples according to their NaCl concentration. An overview of figure 2, it is a clear differences in the first peak at 4.8° for the NSCLC and normal individuals. The calculated parameters are tabulated in table 2; which contains the full width at half maximum of peak 1 ($FWHM_1$) for the NSCLC group is significantly the highest value compared to normal and high risk groups. While the difference between normal and high risk group isn't significant. Moreover, there is a significant shift the first scattering peak position from 4. 8° for healthy individuals, to 4.9° for high risk group, up to 5.1° for the NSCLC samples. While the shift difference in high risk and NSCLC is non-significant (table 2).

The percentage of the amplitude ratio of first to second peaks ((I_1/I_2 %) founds a few decrease in its value in NSCLC and high risk groups compared to healthy group. Only the significant difference is between healthy compared with high risk groups. Peak 1 consists of raising edge (A_1) and falling edge (A_2), the resulting dividing ratio (A_2/A_1) was mentioned in percentage form at table 2. This peak has a unique behavior concerned with its amalgamable of the falling edge of the first peak with the second scattering peak that affect the height A_2 is lower than A_1.

The percentage ratio of (A_2/A_1) represents a significant differences between normal and NSCLC groups with high standard error. While the high risk group percentage ratio of (A2/A1) was in between the normal and NSCLC groups and has a significant difference comparing with the healthy group ; represents in table 2. The shape of the first scatting peak was illustrated in figure 2, it realizes that, its shape is different in all NSCLC samples compared to the normal samples.

The first scattering peak was distorted which is clearly observed in all NSCLC profiles, regardless the varieties of peak shape distortion in each individual samples, it was commonly observed in all NSCLC profiles a decrease in the area under the first peak compared to health group profile. Moreover, area under peak which is known as counts under peak 1, was significantly decreased in NSCLC compared to health group, this parameter is tabulated and represented in table 2.

The substantial finding in this research is concise as a following; a significant six characterization parameters can be discriminated two of the three investigated groups. While (FWHM2) was have a non-significant diversity between all groups (refer to table 2). The significant differences between the normal and NSCLC groups in the parameters; $FWHM_1$, peak position 1, (I_1/I_2 %), (A_1/A_2 %) and counts under peak 1) were found. While the parameters could be using to a significantly discrimination between healthy and high risk groups were two parameters; peak position 1 and (A_2/A_1%), and three parameters elucidated significant differences between NSCLC and high risk groups ; $FWHM_1$, peak position 2 and counts under peak 1. The alterations found in the characterized parameters chiefly the first peak of scattering in all groups could be refer to the previous researches that confirmed the sensitivity of this peak toward the alterations of protein structure [10, 11]. It was previously published; the scattering profile of irradiated serum was have a distortion in their first scattering peak. Obviously, there is no statistical correlation among observed differences in the characterized parameters as a result of the age of the three groups individuals .

LAXS technique beats on the main clinical problem arises in the diagnosis and treatment of NSCLC; which is resulting from lung tissue motions during breath that causing a variations density, hence, it gives inaccurate data especially in early stage (tumor location and size) [11]. To solve this clinical problem, four dimensions computed tomography (4D CT) technique is used to capturing respiratory motion, creating an individual

dynamic data for each patient. The correlation between analyzed 4DCT images in NSCLC patient and his tumor size is significantly proved in its location and classified clinical stage [12, 13]. In contrary to the 4DCT sophisticated procedures and analysis, the LAXS technique is predominant in the simplicity, no radiation hazard for patient, moreover, the high level of accuracy which is independent on the tumor location and size.

4. Conclusion:

According to the National Institutes of Health definition; LAXS could be used as a good biomarker due to it has an objectively measurable characterization, able to evaluate, has a unique indicator of normal biologic processes, ability to recognize pathogenic processes. Moreover, it could be improved the clinical decision-making [13]. Not only all those previously mentioned criteria but also LAXS technique is cheap, specific, durable technique with a great advantage that avoids the radiation hazard through ordinary radiologic investigations for NSCLC patients.

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Conflicts of Interest: The authors declare that, there is no conflict of interest.

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