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ORIGINAL ARTICLE

Establishment of Reference Intervals for Serum Cytokeratin-19 Fragment and Neuron Specific Enolase by Indirect Method Using Data Obtained from Healthy Chinese Population in Chengdu

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SUMMARY

Background: **In order to establish suitable reference intervals (RIs) of serum cytokeratin-19 fragment (Cyfra211) and neuron specific enolase (NSE) for the healthy Chinese population in Chengdu, China, an indirect method was developed using the data from the people presented for routine health check-up.**
Methods: **All results for 4,988 healthy persons serum cytokeratin-19 fragment and 3,293 healthy persons neuron specific enolase were collected in our laboratory information system between January 2016 and December 2018. Outliers were identified and excluded using the stem-and-leaf and box plot methods. Mann-Whitney *U* test was used to observe the difference between sexes. Spearman's rank correlation analysis was used to evaluate the correlation between serum results and age. The RIs were defined by nonparametric 95th percentile interval.**
Results: **After statistical analysis the indirect RIs were 0.0 - 3.70 ng/mL (Cyfra211) and 0 - 17.26 ng/mL (NSE) in males and 0.0 - 3.35 ng/mL (Cyfra211) and 0.0 - 16.29 ng/mL (NSE) in females. Cyfra211 and NSE levels in males and females had no correlation with age. Therefore, there was no need to establish RIs according to age group. RIs of Cyfra211 and NSE were verified and passed the verification in the end.**
Conclusions: **Using health check-up persons' laboratory data values is a relatively easy and cheap method of establishing laboratory specific references. This method deserves to be promoted and applied by other clinical laboratories.**
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INTRODUCTION

Noncommunicable diseases (NCDs) are now responsible for the majority of global deaths, and cancer is expected to rank as the leading cause of death [1]. The American Cancer Society (ACS) released its latest global cancer statistics report in 2018. This report indicates there will be an estimated 18.1 million new cancer cases and 9.6 million cancer deaths in 2018. In both sexes

combined, lung cancer is the most commonly diagnosed cancer (11.6% of the total cases) and the leading cause of cancer death (18.4% of the total cancer deaths) [2]. Although a significant amount of progress has been made in chemotherapy and surgical treatment, the prognosis of this disease remains poor [3]. Therefore, early detection of lung cancer is particularly important, and the detection of a lung cancer biomarker is an effective and common approach by which individuals can be screened for lung cancer. Serum cytokeratin-19 fragment (Cyfra211; a fragment of cytokeratin subunit 19) protein over-expression has been observed in lung cancer and especially in squamous cell carcinoma [4]. Neuron specific enolase (NSE) is a highly specific marker for neuronal and peripheral neuroendocrine cells and is elevated in the serum of patients with small cell lung cancer [5]. So, diagnosis of lung cancer by serum levels of Cyfra211 and NSE are very important for the physician.

In the process of diagnosis, reference intervals (RIs) are medical decision tools that are provided by a clinical laboratory to aid the physician in differentiating a diseased patient from a healthy individual. It is an important task for a clinical laboratory to establish correct RIs.

The concept of RIs was defined and revised in the publication of common International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and Clinical Laboratory and Standards Institute (CLSI) guidelines (EP28-A3c) in 2008 [6]. In this document, a direct method is usually used to set up new RIs with at least 120 reference individuals and strictly healthy people exclusion standards should be proposed during the process. However, it is also acknowledged that determination of RIs is difficult, time-consuming, and expensive. Therefore, few laboratories in hospitals can develop their own RIs. The new document now allows individual laboratories to adopt RIs established elsewhere by transference and verification. Therefore, in China the RIs used in the laboratory always follow the manufacturers of the diagnostic tests. For the domestic population, many reagents used in the clinical chemistry laboratory are from the United States and Europe and the RIs are based on the data of white population many years ago which probably leads to an inaccurate diagnosis. Variations in the RIs from different populations may especially affect patient management [7]. Therefore, it is necessary to establish our own RIs that are suitable for the local residents [8].

For all these reasons, some scientists working in the area have investigated the possibility of establishing RIs from large collections of laboratory data, using sophisticated laboratory information systems and statistical programs [9,10]. An indirect method of setting up RIs depends on the huge data collected in the laboratory. The major advantage of using such an approach is that it saves a significant amount of money and work compared to the direct method. It is a convenient way for the clinical laboratory to set up its own RIs.

In this study, the indirect method was used to establish serum RIs for Cyfra211 and NSE of people in Chengdu, Sichuan province in China. We also investigated whether there were significant differences in these two proteins between sexes and evaluated the correlations between these tumor biomarker levels and age.

MATERIALS AND METHODS

Subjects

All data of Cyfra211 and NSE were collected from Han population presented for routine health check-up in Chengdu Fifth People's Hospital from January 2016 to December 2018. Screening criteria were developed to make sure that people do not have obvious diseases, no family genetic diseases, no tumors and to ensure that cardiopulmonary function, blood pressure, blood biochemical examination results were normal. No pregnant women were included because they were not suitable for health check-up and were excluded in the initial screening.

After screening, 4,988 serum Cyfra211 and 3,293 serum NSE data were collected in our laboratory information system. All measurements were performed by electrochemiluminescence immunoassay using Roche Cobas E601 system (Roche Diagnostics GmbH, Mannheim, Germany). Two levels of internal quality control were conducted daily and the tests were covered by an external quality assessment scheme (Clinical Test Center of Ministry of Health, China).

All data were analyzed by SPSS 19.0 (IBM Corporation, Armonk, NY, USA) and Kolmogorov-Smirnov test was used to decide whether the data show a normal distribution. If the data are not normally distributed, the values will be transformed to a normal distribution by a logarithmic procedure. In the indirect method, data exclusion always influences the final RIs. Stem-and-leaf and box plots were used to remove the outliers after logarithmic transformation. Mann-Whitney *U* test was applied to analyze the differences between males' and females' results. If the differences are significant, the RIs were then also estimated for men and women separately. In accordance with CLSI EP28-A3c, the RIs were defined by nonparametric 95th percentile intervals. As for tumor biomarker the lower limit of reference is of no clinical significance, so the value of 95% position was used as upper limits. Spearman's rank correlation analysis was used to analyze the correlation between the two biomarkers levels and age. According to CLSI EP28-A3c, RIs of Cyfra211 and NSE were verified.

RESULTS

Kolmogorov-Smirnov test showed a skewed distribution for serum CYFRA211 and NSE levels, so logarithmic procedure was used to transform the data to normal distribution. Then stem-and-leaf and box plots was

Table 1. The data of Cyfra211 and NSE included in study.

Test name	Gender	n	Outliers	Retained data	Age
Cyfra211	male	2,681	74	2,607	48 (17 - 89)
	female	2,307	82	2,225	46 (18 - 85)
Total		4,988	156	4,832	
NSE	male	1,747	54	1,693	46 (20 - 88)
	female	1,546	66	1,480	47 (18 - 85)
Total		3,293	120	3,173	

Table 2. The differences between males' and females' results.

Test name	Male	Female	Sig. (two-tailed)
Cyfra211	1.97 (0.27 - 4.18)	1.73 (0.19 - 3.89)	0.000
NSE	11.24 (5.19 - 17.81)	11.07 (5.14 - 16.69)	0.000

Correlation is significant at 0.05 level.

Table 3. Correlations between Cyfra211 and NSE test results and age.

Test name	Gender	Spearman correlation	Sig. (two-tailed)
Cyfra211	male	0.002	0.194
	female	0.001	0.180
NSE	male	0.002	0.256
	female	0.003	0.233

Correlation is significant at 0.05 level.

Table 4. RIs for Cyfra211 and NSE calculated from health check-up data.

Test name	Gender	Indirect RIs	URL (95% CI)	Manufacturer's RIs
Cyfra211	male	0 - 3.70 ng/mL	3.41 - 3.78 ng/mL	0 - 3.3 ng/mL
	female	0 - 3.35 ng/mL	3.15 - 3.55 ng/mL	
NSE	male	0 - 17.26 ng/mL	16.53 - 18.40 ng/mL	0 - 16.30 ng/mL
	female	0 - 16.29 ng/mL	15.39 - 17.07 ng/mL	

URL - upper reference limit.

used to remove the outliers. The total number of healthy persons included in the study was 4,988 for Cyfra211 and 3,293 for NSE at first, and then 156 of Cyfra211 and 120 of NSE results were excluded because they were detected as outliers after statistical treatment. Therefore, 4,832 of Cyfra211 and 3,173 of NSE results were retained and analyzed (Table1).

For two subclasses (male and female), the statistical significance of the difference between subclass means was tested by Mann-Whitney *U* test. Significant difference

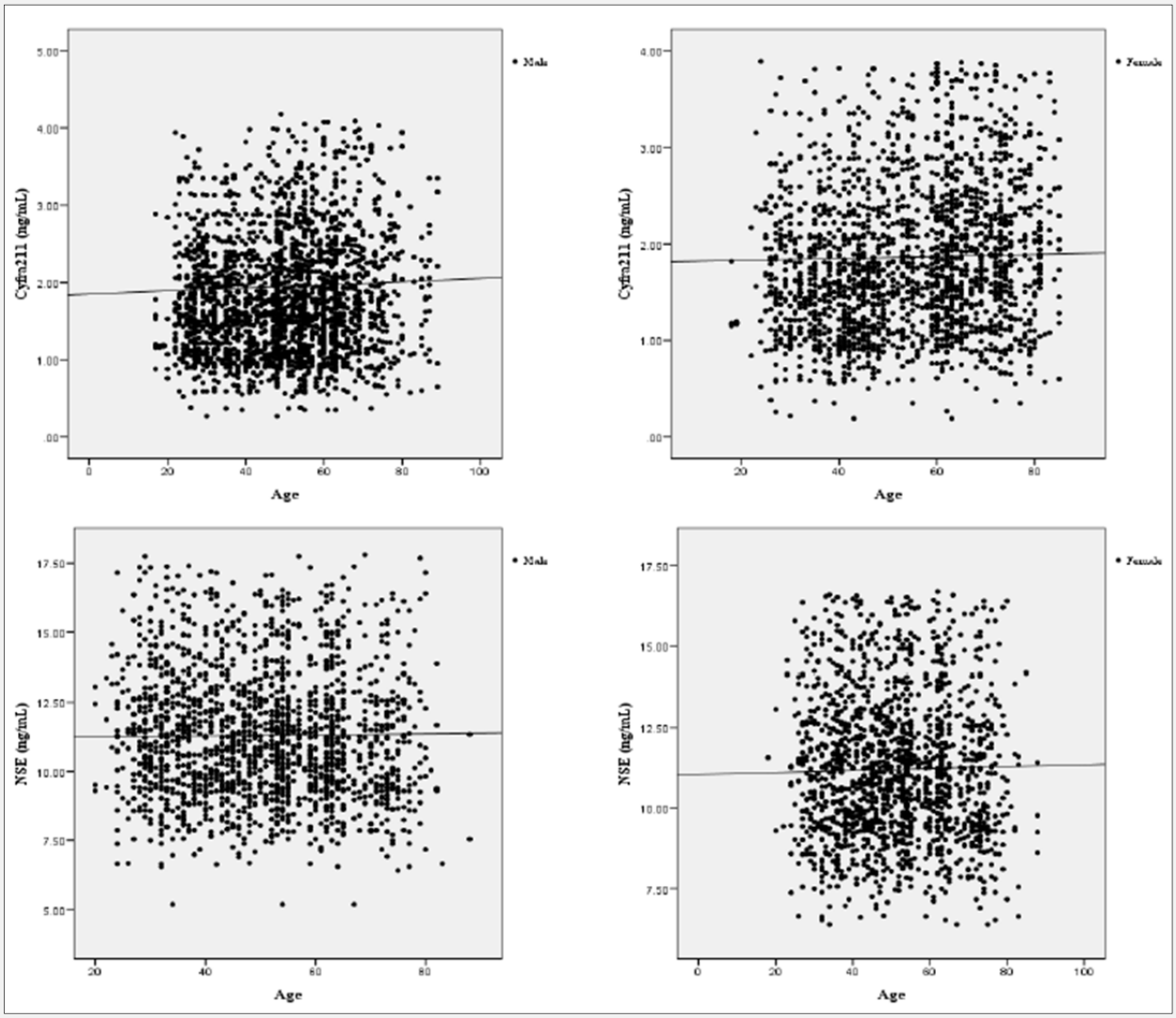

Figure 1. Scatter plots of Cyfra211, NSE levels, and age. Regression curve was also plotted.

was found between serum Cyfra211 and NSE concentrations in male versus female participants (Table 2). Scatter plots of Cyfra211, NSE levels, and age are shown in Figure 1. Correlations between biomarker levels and age were analyzed by Spearman's rank correlation analysis (Table 3). There was no significant correlation between Cyfra211, NSE levels, and age.

RIs (95% confidence interval: 95% CI) for Cyfra211 and NSE were calculated by nonparametric 95th percentile intervals from health check-up data (indirect method) and compared them with the RIs recommended by the manufacturer (direct method) (Table 4). In addition, RIs of Cyfra211 and NSE were verified and passed the verification in the end.

DISCUSSION

To improve the diagnosis of lung cancer, many tumor markers, including Cyfra211 and NSE, have been intensively evaluated and widely used in the diagnosis. However, each marker has its own RI, which is based on those established from US or European persons and might generate the confusing results because differences between populations are an issue and can reduce the validity of reference values [11]. So, it is vital that each laboratory should have its own RIs based on the analysis system.

Although direct method is usually used to set up new RIs, the complexity of establishing reference intervals

and the cost and labor are additional difficulties. These factors have forced clinical laboratories to use other methods to develop laboratory specific RIs. In our laboratory information system, we have a large number of useful data and, thus, the indirect method can be properly used to set up our Cyfra211 and NSE RIs. In this study, we collected health check-up data. The cohort did not have obvious diseases but were there for their annual examination. No pregnant women were included because there is an X-ray examination in health check-up and pregnant women were excluded first. Finally, 4,988 of Cyfra211 and 3,293 of NSE data were included which are partly representative of health check-up results of apparently healthy people.

Before statistical analysis, we needed to confirm whether the data were normally distributed. The Kolmogorov-Smirnov test showed that the data were not normally distributed. Logarithmic procedure is a common method that is often used to transform data. The elimination of outliers always influences the final RIs by the indirect method, and these results must be sorted out. It is therefore important to adopt the proper method. After the data were converted into a normal distribution, the box plot method was used to remove the outliers. The software identifies outliers first by computing the interquartile range (IQR) between the lower and upper quartiles of the distribution and then by determining the data lying outside 3 IQRs from the upper or lower edge of the box. The procedure was repeated until no extremes were left.

Many laboratories use the same RIs of NSE and Cyfra211 for males and females. But in our study, there were significant differences between genders. So, we established RIs for Cyfra211 and NSE separately according to the gender. In addition, according to CLSI EP28-A3c, RIs of Cyfra211, and NSE were verified and passed the verification in the end.

In a previous study, the RI of NSE was based on foreign population and used a different diagnostic system [12]. There has been no report using the Roche Diagnostic System to establish a reference value for NSE for a Chinese population. Compared to the manufacturer's RI (0 - 16.30 ng/mL), our new RI for NSE in males (0 - 17.26 ng/mL) has a wide range and is almost identical to that in females (0 - 16.29 ng/mL). In addition, it has a higher value in the upper limit of males compared to females which may be related to lifestyle or physiological differences.

For Cyfra211 the RIs in our study were 0 - 3.70 ng/mL in males and 0 - 3.35 ng/mL in females, higher than that recommended by Roche Diagnostics GmbH (3.3 ng/mL) which was established in a western population. In other study using the Roche diagnostic system, Woo HY [13] established the RI for Cyfra211 in healthy Korean adults (0 - 3.59 ng/mL) several years ago and Bing Zhao [14] also recommended the RI for Cyfra211 (0 - 4.47 ng/mL) in Taizhou city of China. In a recent study, the RI for serum Cyfra211 had been established in Chinese population and the upper limit of the 97.5th percentile was 3.55 ng/mL [15], but the diagnostic system was ARCHITECT immunoassay (Abbott Diagnostics). Therefore, the difference may be due to different race and geography of the selected population and different diagnostic system. Our established RI for serum Cyfra211 will be more applicable for Chengdu population.

No significant correlation between NSE, Cyfra211 levels, and age was found in males and females. So, there is no need to set up different RIs of NSE and Cyfra211 based on ages. This is consistent with the RIs provided by the instrument manufacturer.

After the RIs of serum Cyfra211 and NSE were established, one important question was whether the established RIs were suitable for our laboratory. Because it is the responsibility of clinical laboratories or laboratory networks to use RIs that are appropriate for their methodologies and the population they serve. Therefore, the established RIs must be verified. The standard approach to verify RIs recommended by the CLSI EP28-A3c guideline for routine clinical laboratories is to collect and analyze samples from 20 healthy subjects per age and/or gender partition from the receiving laboratory's local population and to compare these values with the established RI. If no more than 2 of the 20 samples fall outside the RI, it may be received for use. So RIs of Cyfra211 and NSE were verified separately by gender grouping and passed the verification in the end. Although we have established the RIs for Cyfra211 and NSE, there are still some limitations in this study. This is a single-center and single-ethnic group study suitable for Han ethnicity in Chengdu area, which may make the results less representative. Because in Sichuan province including Chengdu area, there are many ethnic groups, with the largest being Tibetan. So, further multicenter and multi-ethnic studies are needed. In addition, our measurements for serum Cyfra211 and NSE were conducted on the Roche Cobas E601 system by electrochemiluminescence immunoassay. Therefore, the RIs for NSE and Cyfra211 in other detection systems should also be established. Since laboratory methods can affect the RIs, our established RIs for serum NSE and Cyfra211 could be different from those by other systems using other measurement methods [16-18].

CONCLUSION

In summary, establishing RIs from local health check-up population's results may be more suitable for the laboratory, and the indirect method is the cheapest and easiest way. Because they are derived from local residents with the same conditions, they are likely to match clinical results better.

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Declaration of Interest:
The authors report no potential conflict of interests.

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