Circulating Cysteine Rich Protein 61 (Cyr61, CCN1) in Chinese Adults: Distribution and Reference Intervals

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SUMMARY

Background: The circulating levels of Cyr61 (also known as CCN1) may prove to have great clinical value in the diagnosis, monitoring and prognosis of many disorders in humans. However, the reference intervals (RIs) for this analyte in human subjects have not previously been well established. Therefore, establishing RIs and determining the distribution of circulating Cyr61 levels are very important for future clinical studies and could provide an orientation value for exploring its clinical usefulness.

Methods: The Cyr61 levels in 2,514 healthy Chinese Han subjects (1,250 males and 1,264 females, aged 18 - 88 years, recruited from 4 hospitals in Shanghai and Fujian) were measured with a sandwich ELISA (R&D Systems, USA). The RIs were determined in a manner consistent with the Clinical and Laboratory Standards Institute guidelines.

Results: The levels of serum Cyr61 showed a non-Gaussian distribution. A statistically significant difference was observed between the males and females such that the median level of Cyr61 in the males was significantly higher than that in the females. Furthermore, the Cyr61 levels significantly increased with age in the female group whereas no difference was observed among the different age groups among the males. The RIs for serum Cyr61 were 3.3 - 184 pg/mL and 5.0 - 182 pg/mL in females aged 18 - 45 and 46 - 88 years, respectively. The RI for serum Cyr61 was 4.0 - 198 pg/mL in the males.

Conclusions: The RIs for serum Cyr61 were established among Chinese Han individuals. The effects of age and gender on the distribution characteristics of serum Cyr61 were studied, revealing that the RIs were gender and, in females, age-specific, which may suggest that a female hormone, estrogen plays a role in the regulation of Cyr61 expression in vivo.


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INTRODUCTION

Cysteine rich protein 61 (Cyr61), also called CCN1 as a member of the CCN family, is a heparin-binding protein encoded by an immediate early gene (IEG) [1]. The CCN family comprises CCN1 to CCN6 [2]. As a secreted extracellular matrix (ECM) protein, Cyr61 is critically involved in embryonic development, wound healing, tissue remodeling, angiogenesis, and osteoblast differentiation. By binding integrin receptors (such as αvβ3, αvβ5, αvβ6, and α6β1) expressed on the cell surface [1], Cyr61 can regulate cell adhesion, migration, proliferation and neovascularization in cell type-specific and function-specific manners [3]. Increased Cyr61 is associated with many disorders in humans. In malignancies (such as breast cancer, esophageal squamous cell carcinoma, and lung cancer [4-6]), Cyr61 expression was found to be dysregulated. Furthermore, overexpressed Cyr61 in pathogenic tissue cells has been found in atherosclerotic plaque formation and cardiovascular diseases [7]. In dysfunctional pregnancy, a decreased Cyr61 expression is consistent with abnormal placental development and function [8].

Recently, several studies reported that Cyr61 could be produced by immune cells and attracts immune cells to inflammatory sites, thereby aggravating inflammation and tissue damage [9], suggesting that Cyr61 plays an initial role in the inflammation process. Accumulating data have also indicated that in inflammatory environments, tissue cells (e.g., synoviocytes in rheumatoid arthritis and keratinocytes in psoriasis) have increased concentrations of Cyr61 protein. In addition, the serum concentrations of Cyr61 have been found to be positively correlated with complement 4 (C4) in patients with systemic lupus erythematosus (SLE) [10], indicating that changes in the serum Cyr61 concentrations might be helpful for disease diagnosis and prognosis.

Given the potential role of Cyr61 in the process of diseases, quantifying the serum concentrations of Cyr61 in a multicenter and relatively large normal human population is an essential requirement for evaluating Cyr61 as a biomarker of pathogenesis. However, most studies investigating the Cyr61 levels in normal and disease subjects are based on a relatively small specimen library. Moreover, studying the factors affecting normal serum Cyr61 levels and their distribution characteristics is crucial for establishing reference intervals (RIs) for future clinical applications.

In this study, we are the first to measure the normal serum Cyr61 level in 2,514 healthy adults and analyze the distribution characteristics of serum Cyr61. Our study shows the distribution characteristics and RI range of normal serum Cyr61 concentrations in a healthy adult population. These results provide a very important reference for studies investigating the relationship between serum Cyr61 levels and some human diseases. Moreover, readily available normal serum levels of Cyr61 could promote studies exploring Cyr61 in the pathogenesis and progression of human diseases, thereby resulting in a better interpretation for future clinical applications.

MATERIALS AND METHODS

Study population

Chinese Han participants were recruited during a physical examination at Shanghai Tongren Hospital, Shanghai 10th People’s Hospital, Shanghai Ruijin Hospital, and the 1st Affiliated Hospital of Fujian Medical University. The health status of the participants was determined by a self-report questionnaire and laboratory examinations. All participants met the following requirements: no history of cardiovascular disease, diabetes mellitus, autoimmune diseases, or cancer; not taking lipid-lowering drugs or corticosteroids; high density lipoprotein cholesterol (HDL-c) level above 0.91 mmol/L and triglyceride, total cholesterol, low density lipoprotein cholesterol (LDL-c), and fasting blood glucose levels below 1.70 mmol/L, 5.70 mmol/L, 3.61 mmol/L, and 6.16 mmol/L, respectively; normal regular blood test results; and normal liver and kidney function test results.

Participants with incomplete information or abnormal laboratory indicators were excluded. Ultimately, 2,514 healthy subjects aged 18 - 88 years were enrolled, including 1,250 males and 1,264 females. Ethical approvals for the study and the use of human subjects were obtained from the research ethics committee of the hospitals and were consistent with the ethical guidelines of the 1975 Declaration of Helsinki.

Sample collection

All samples were drawn by venipuncture and transported and processed according to the Guidelines for the Collection and Transportation of Samples for Testing (PUMCHL-L-2-Q25b-04) program. Venous blood was collected from the subjects in a quiet and fasting state. The serum samples were obtained by centrifugation within 2 hours of the initial collection and stored at -80°C for processing. Routine analyses of blood samples were completed on the same day.

Cyr61 assay

The concentration of serum Cyr61 was detected by using a sandwich ELISA (DY 4055, R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Calibrators and controls were run in duplicate. One in-house serum pool used as a control was included in each run. A standard curve was generated for each plate and used to calculate the concentration of each sample. The precision (CV%) of the assay was 9% at 3.1 pg/mL, 8% at 26 pg/mL, and 3% at 200 pg/mL.
The LOD and LOQ were 0.6 pg/mL and 2.5 pg/mL, respectively.

**Statistical analyses**
All experimental data were statistically analyzed using SPSS 17.0 software (IBM Inc., NY, USA), GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and/or MedCalc Statistical software 15.0 (Mariakerke, Belgium). The statistical methods recommended by the Clinical and Laboratory Standards Institute (CLSI) EP-28-A3c document were used to determine the central 95% RIs [11]. The non-Gaussian distributed data are presented as the medians (quartiles). The interquartile ranges were also calculated, and non-parametric analyses were applied to calculate the median and 2.5th and 97.5th percentiles (P2.5 - P97.5). Kolmogorov-Smirnov tests and logarithmic transformations were used to evaluate the distributions of the data. Kruskal-Wallis tests (non-parametric tests) were used for all comparisons among different groups. For the comparisons between the gender groups, Mann-Whitney tests were used. P-values below 0.05 indicated statistical significance.

**RESULTS**

**Determination of the normal levels and distribution characteristics of serum Cyr61 in humans**
The normal serum Cyr61 levels in a total of 2,514 Chinese Han participants are shown in Figure 1A; the data are presented as the medians with the 25th to 75th percentiles due to the non-Gaussian distribution. The serum levels of Cyr61 were 42.5 pg/mL (22.0 to 75.4 pg/mL) in the total sample, 45.7 (23.1 to 76.3 pg/mL) in the males and 39.0 (21.4 to 72.5 pg/mL) in the females (Figure 1).

We further analyzed the difference in the normal serum Cyr61 level between the males and females. As shown in Figure 1B, the normal serum Cyr61 level in the males was higher than that in the females (45.7 (23.1 to 76.3 pg/mL) vs. 39.0 (21.4 to 72.5 pg/mL)). In fact, the difference between the males and females was statistically significant (p < 0.01).

**Gender and age influence the normal serum Cyr61 levels**
The distribution characteristics of the normal serum Cyr61 levels were studied to reveal the influencing factors by analyzing the frequency profiles of the normal serum Cyr61 levels in 2,514 Chinese Han participants. The normal serum Cyr61 level showed a non-Gaussian distribution. The 2.5th to 97.5th percentiles of serum Cyr61 was 4.0 to 193 pg/mL.

To investigate whether gender is a possible factor influencing the non-Gaussian distribution, we performed a statistical analysis. The normal serum levels of Cyr61 revealed a non-Gaussian distribution in both the males and females. The 2.5th to 97.5th percentiles of the serum Cyr61 levels in the males and females were 4.0 to 198 pg/mL and 4.2 to 190 pg/mL, respectively. However, a Gaussian distribution was observed in the females (p = 0.191) after a logarithmic transformation whereas a non-Gaussian distribution of the normal serum Cyr61 level was still observed in the total sample (p < 0.001) and the males (p < 0.001) as shown in Figure 2. Taken together, the distribution characteristics of normal serum Cyr61 levels in females were different from those in males, which implied that gender might have influence on the normal Cyr61 serum level.

To investigate whether age is a possible factor influencing the non-Gaussian distribution, we performed a statistical analysis as shown in Figure 3. The participants were divided into different groups based on age (18 - 25, 26 - 35, 36 - 45, 46 - 55, 56 - 65, 66 - 75, and > 75 years) in male and female groups. The normal serum Cyr61 levels among the females showed statistically significant differences across the seven groups (Figure 3B, p = 0.027). However, the males did not show any statistically significant differences among the seven age groups (p = 0.413, Figure 3A).

The post hoc analysis revealed that for females there were no statistically significant differences among the younger three groups (≤ 45 years) or the four older groups (> 45 years); thus, the female participants were combined into two groups, i.e., the younger group (≤ 45 years) and older group (> 45 years), as shown in Figure 3C. The serum Cyr61 levels in the younger group were significantly lower than those in the older group (p < 0.001) and the male group (p < 0.001).

In summary, significant differences in distribution characteristics of the Cyr61 serum levels between the female and the male were observed. These results suggested that the Cyr61 serum levels were gender-dependent. An age-specific distribution was only found in females.

**Establishment of RIs**
In this study, the RIs (2.5th and 97.5th percentiles) and the 90% CIs of serum Cyr61 derived from 2,514 Chinese adults were calculated using the non-parametric method. The RIs were established based on gender and age because gender and age influenced the distribution of the normal serum Cyr61 level. As shown in Table 1, the RIs for serum Cyr61 were 3.3 (90% CI: 2.5 - 5.2 pg/mL) to 184 pg/mL (90% CI: 156 - 216 pg/mL) and 5.0 (90% CI: 4.0 - 6.5 pg/mL) to 182 pg/mL (90% CI: 168 - 223 pg/mL) among the females aged 18 - 45 and 46 - 88 years, respectively (Table 1). The RI for serum Cyr61 was 4.0 (90% CI: 2.0 - 5.0 pg/mL) to 198 pg/mL (90% CI: 179 - 231 pg/mL) in the males because there was no statistically significant difference among the different age groups. These results indicate that establishing RIs for serum Cyr61 based on the variation in different gender and age groups in females is necessary.
Table 1. Reference intervals for serum Cyr61 (pg/mL) in healthy Chinese participants calculated by the non-parametric method.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age (years)</th>
<th>n</th>
<th>Lower reference limit (2.5th percentile)</th>
<th>90% CI *</th>
<th>Upper reference limit (97.5th percentile)</th>
<th>90% CI *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>18 - 45</td>
<td>677</td>
<td>3.3</td>
<td>2.5 - 5.2</td>
<td>184</td>
<td>156 - 216</td>
</tr>
<tr>
<td></td>
<td>46 - 88</td>
<td>587</td>
<td>5.0</td>
<td>4.0 - 6.5</td>
<td>182</td>
<td>168 - 223</td>
</tr>
<tr>
<td>Male</td>
<td>18 - 86</td>
<td>1,250</td>
<td>4.0</td>
<td>2.0 - 5.0</td>
<td>198</td>
<td>179 - 231</td>
</tr>
</tbody>
</table>

CI * - confidence interval.

Figure 1. Distribution of normal serum Cyr61 levels in Chinese Han participants.
A: Dot plot of the normal serum Cyr61 levels in Chinese Han participants by gender. The center lines represent the median, and the top and bottom lines represent the 75th and 25th percentiles, respectively. B: Difference in the normal Cyr61 serum levels between males and females. The boxplot represents the median, 75th percentile, 25th percentile, and maximum and minimum values of the serum Cyr61 levels. Mann-Whitney tests were used for the comparisons between the gender groups. "**" - p < 0.01.

DISCUSSION

Due to the potential role of Cyr61 in the pathogenesis and progression of human diseases, estimating the reference serum level of Cyr61 in clinical studies is essential. Serum is well known to be the most readily available type of clinical sample. However, thus far, most clinical data of Cyr61 have been derived from changes in the Cyr61 expression level in tissues [12-14], limiting large-scale studies and the understanding of the role of Cyr61 in disease pathogenesis in human.

RIs are very important for clinical studies. RIs serve as a criterion for any test result to provide the information necessary for clinical diagnosis and treatment [15-17]. To the best of our knowledge, this study is the first to establish RIs for serum Cyr61 levels in healthy Chinese Han participants [12] following the CLSI EP28-A3c guidelines.

In this study, we provided gender- and age-specific RIs for serum Cyr61 derived from 2,514 Chinese adults. Differences in the RIs for serum Cyr61 between the male and female groups and different age groups in the female group were reported.

Given that Cyr61 has been described as an estrogen target gene in the myometrium and breast cancer [18], the expression of Cyr61 in tissue and the concentration in serum might be affected by age and gender. In fact, we found that the normal serum Cyr61 level in the females in the younger group (≤ 45 years) was significantly lower than that in the older group (> 45 years), which is consistent with female hormone levels. Moreover, there were no statistically significant differences in the males
among the different age groups. This finding suggests that the circulating levels of Cyr61 in women may be partially negatively regulated by the female hormone levels, particularly estrogen. Consequently, there was no significant difference between the females and males in the older group. Nevertheless, extending our study by elucidating the RIs in different female groups with a large sample size and different ethnic populations is important to further confirm the observation. Furthermore, some modification of the RIs may be needed for other clinical assay methods because the RIs and the distributional characteristics in this study were determined with ELISA.
In addition, estrogen is thought to play a protective role in females against certain types of diseases, especially female tumors [19, 20]. Our study reveals that estrogen may contribute to the variation in the normal serum Cyr61 levels among different ages in females and further supports the finding that estrogen might negatively regulate Cyr61 expression in vivo.
Estrogen production begins to decline in females while aging, whereas the normal serum Cyr61 level begins to increase along with increased incidences of female tumors, which suggest the Cyr61 plays a role in female tumorigenesis. Indeed, an increase of the Cyr61 serum level in most of female tumors was reported [3, 4]. Therefore, monitoring the changes in the serum Cyr61 levels may be useful for stratifying the risk for certain female tumors. Establishing the RIs for serum Cyr61 based on different ages and gender is crucial for future clinical studies and could provide an orientation value for exploring the clinical usefulness of such measurements.

**Figure 2. Frequency profiles (histograms) of the normal serum Cyr61 levels.**

A: Frequency profiles (histograms) of the normal serum Cyr61 levels in the total sample, the male and the female groups. B: Frequency profiles (histograms) with a logarithmic transformation in the total sample, the male and the female groups. Superimposed curves in both A and B are Gaussian curves based on the data set’s mean and SD.
Figure 3. Distribution of normal serum Cyr61 levels in different age groups.

A: Normal serum Cyr61 levels in seven different age groups of males. B: Normal serum Cyr61 levels in seven different age groups of females. C: Differences in the normal serum Cyr61 levels among the female younger group, female older group, and male group. The vertical lines shown within each box indicate the median values; the left and right edges of the boxes indicate the 25th and 75th percentiles, respectively; and the whiskers show the full range of the data (from minimum to maximum). Kruskal-Wallis tests were used for the comparisons among the different groups, and Mann-Whitney tests were used for the comparisons between two groups. ***p < 0.0001.

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Declaration of Interest:
The authors declare that they have no conflicts of interest.

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