Alterations in CD8⁺ Tregs, CD56⁺ Natural Killer Cells and IL-10 Are Associated With Invasiveness of Nonfunctioning Pituitary Adenomas (NFPAs)

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Invasive nonfunctioning pituitary adenomas (NFPAs) grow rapidly and the mechanisms are unclear. Among many complex mechanisms, the role of immunity in the development of NFPAs has not been fully explored. Here, we analyzed the clinical features 146 NFPAs patients who underwent trans-sphenoidal surgery or craniotomy and examined the effects of immune tolerance in invasiveness of NFPAs patients using fluorescence-activated cell sorting and immunohistochemical methods. We found patients with invasive NFPAs had more visual deficits and defective fields, higher tumor size, and lower white blood cell count compared with patients with noninvasive NFPAs. Additionally, compared with patients with noninvasive NFPAs, patients with invasive NFPAs had conspicuously lower CD3⁻CD56⁺ natural killer (NK) cells and significantly higher levels of CD3⁺CD8⁺CD28⁻ T-cells (CD8⁺ Tregs) and interleukin-10 (IL-10) in peripheral blood. Moreover, patients with invasive NFPAs had lower infiltrated CD56⁺ cells, less infiltrated CD28⁺ cells, and significantly greater IL-10 expression. These results demonstrated that low CD56⁺ cells infiltration and CD28⁺ cells infiltration, as well as high IL-10 expression in pituitary tumor tissues, were related with increased invasiveness of NFPAs. Levels of CD3⁺CD56⁺ NK cells, CD8⁺ Tregs and IL-10 in the peripheral blood could be feasible diagnostic markers for invasive NFPAs.

Keywords: invasive nonfunctioning pituitary adenomas, immune tolerance, CD8 + tregs, natural killer cells, IL-10

INTRODUCTION

Pituitary adenomas (PAs) account for 10–25% of all intracranial neoplasms [1]. They are derived from intracranial adenohypophyseal cells and often presented as neurological deficits (particularly visual impairment), pituitary dysfunction, parasellar compartment invasion and sphenoid sinuses [2]. Although the majority of PAs are histologically benign, 25–55% of PAs often invade surrounding structures and exhibit malignant behaviors [3, 4]. Up to now, neurosurgery resection remains the initial treatment of choice for most PAs. However, curative radical surgery of invasive PAs remains difficult. Furthermore, approximately 10–20% PAs are unable to produce active hormone and classified as nonfunctioning pituitary adenomas (NFPAs) [5]. As hormone inactivity leads to delayed
diagnosis, the incidence of invasive NFPAs becomes higher. In addition, the efficiency of NFPAs chemotherapy is unsatisfactory and there is no consensus regarding the radiotherapy. Therefore, it is urgent to elucidate the mechanisms of the biological behavior of PAs, especially for NFPAs, thereby developing an effective treatment for them [6].

Immune tolerance or escape is pivotal in tumor development, progression, and control [7, 8] and mediated by both deletion of self-reactive T cells and activation of suppressing T-cell activity [9]. Studies of brain tumor-infiltrating lymphocytes (TILs) have provided evidence demonstrating that the immune system is naturally involved in the immune surveillance of brain tumors [10–12]. Pituitary adenomas are the second most common type of intracranial tumor. Recently, pituitary adenomas have been found [13–16] to have varying degrees of immune cell infiltrates. Greater infiltration of CD68+ macrophages could increase the production of CD4+ regulatory T cells (Tregs) to suppress the immune system [17, 18] and is associated with increased invasiveness of adenomas [16]. The study have demonstrated that down-regulation of transforming growth factor-beta (TGFβ, one of the main immune suppressive mediators) is correlated with tumorigenesis process of NFPAs [19]. However, the role of immunity in the development of NFPAs has not been fully explored. Accordingly, in this study, we examined the clinical characteristics of patients with NFPAs and assessed the effects of inflammatory or immune cells on invasive NFPAs.

METHODS

Participants and Samples
A total of 146 patients with NFPAs who underwent transsphenoidal surgery or craniotomy and 20 healthy controls at the Fifth People’s Hospital of Shanghai, Fudan University between 2012 and 2016 were included in this study. Age and sex-matched 20 normal subjects were selected from physical examination center of our hospital. Patients who had undergone previous drug therapy or radiation therapy or had a recurrence or pituitary apoplexy were excluded. Patients were also excluded if they were taking medications that could influence immune function, such as glucocorticoids and immunosuppressants. Patients with obesity, diabetes mellitus, severe hypertension, infectious diseases, severe hepatic and renal dysfunctions and other malignant tumors were also excluded. The diagnosis of NFPAs was based on clinical symptoms and signs including amenorrhea, oligomenorrhea, galactorrhea, infertility, hirsutism, acne, enlargement of the hands and feet, headaches, and vision loss as well as hormonal levels, magnetic resonance imaging, histopathological examination, and immunohistochemical staining for all anterior pituitary hormones. To evaluate changes in the percentages of lymphocytes and levels of cytokines, 2 ml of ethylenediaminetetraacetic acid-anticoagulated whole blood and 4 ml of procoagulant serum were collected from the 146 patients with NFPAs and 20 healthy controls. The surgical adenomas specimens were also obtained from the 146 patients with NFPAs to assess the infiltration of lymphocytes, and expression of inflammatory cytokines in adenomas. The study protocol was approved by the Ethics Committee of Shanghai Fifth People’s Hospital, Fudan University (2014029), and all participants gave written informed consent to participate in the study in accordance with the Declaration of Helsinki.

Pituitary Imaging, Hormone Analysis, and Cytokine Testing
MRI was used to measure the diameters of NFPAs and evaluate tumor invasion. Tumor invasiveness was assessed based on Hardy’s classification. Only grade III and IV tumors were considered as invasive NFPAs. Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), testosterone (T) and prolactin (PRL) were examined using electrochemiluminescence immunoassays and serum levels of growth hormone (GH), thyroid-stimulating hormone (TSH), free thyroxine (FT4), adrenocorticotropic hormone (ACTH), and cortisol (C) were examined using chemiluminescence assays. Serum levels of interferon-γ (IFN-γ), interleukin-2 (IL-2), and interleukin-10 (IL-10) were measured by enzyme-linked immunosorbent assay (ELISA; Raybio) according to the manufacturer’s protocols.

Lymphocyte Subtype Analysis by Fluorescence Activated Cell Sorting
The T-cell subtypes were determined using FACS analysis. In brief, whole blood samples were lyzed with Lymphoprep™ (STEMCELL Technologies, Vancouver, Canada). Lymphocytes were stained with 100 μL phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% NaN3, together with 5 μL PerCPcy5.5-conjugated anti-human CD3 (cat. no. 45-0037-42), followed by simultaneous staining with 5 μL fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (cat. no. 11-0049-42) or APC-conjugated anti-human CD8 (cat. no. 17-0086-42) and phycoerythrin (PE)-conjugated anti-human CD28 (cat. no. 12-0289-42) or FITC-conjugated anti-human CD56 (cat. no. 11-0566-42; all eBioscience). The cells were then incubated for 20 min at 4°C, and flow cytometry was performed using a BD FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, United States).

Immunohistochemistry
The surgical specimens were processed to 5 μm sections using conventional formalin-fixed, paraffin-embedded method. These sections were dewaxed, boiled for 20 min in citrate buffer (10 mM, pH 6.0) to retrieve antigens, and subjected to immunohistochemical staining for CD3 (cat. no. GB13014; antibody dilution 1:50; Servicebio), CD4 (cat. no. GB13064-1; antibody dilution 1:25; Servicebio), CD8 (cat. no. GB13068; antibody dilution 1:50; Servicebio), CD28 (cat. no. ab113358; antibody dilution 1:100; Abcam), CD56 (cat. no. 14255-1-Ap; antibody dilution 1:100; Servicebio), IL-10 (cat. no. GB13108; antibody dilution 1:400; Servicebio), IFN-γ (cat. no. 5365-1-Ap; antibody dilution 1:100; Proteintech), and IL-2 (cat. no. ab92381; RayBiotech, Inc.) and examined using electrochemiluminescence immunoassays and immunohistochemical staining for all anterior pituitary hormones.

Fluorescence Activated Cell Sorting
The T-cell subtypes were determined using FACS analysis. In brief, whole blood samples were lyzed with Lymphoprep™ (STEMCELL Technologies, Vancouver, Canada). Lymphocytes were stained with 100 μL phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% NaN3, together with 5 μL PerCPcy5.5-conjugated anti-human CD3 (cat. no. 45-0037-42), followed by simultaneous staining with 5 μL fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (cat. no. 11-0049-42) or APC-conjugated anti-human CD8 (cat. no. 17-0086-42) and phycoerythrin (PE)-conjugated anti-human CD28 (cat. no. 12-0289-42) or FITC-conjugated anti-human CD56 (cat. no. 11-0566-42; all eBioscience). The cells were then incubated for 20 min at 4°C, and flow cytometry was performed using a BD FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, United States).
### TABLE 1 | Demographic and clinical characteristics of 146 patients with NFPAs.

|                      | Control (n = 20) | Noninvasive NFPAs (n = 60) | Invasive NFPAs (n = 86) | p value |
|----------------------|------------------|-----------------------------|-------------------------|---------|
| **Age** (years)      | 57.75 ± 1.14     | 57.93 ± 1.86                | 57.56 ± 1.20            | 0.983   |
| **Sex, no. (%)**     |                  |                             |                         | 0.355   |
| Female               | 10 (50.0%)       | 20 (33.3%)                  | 36 (41.9%)              | —       |
| Male                 | 10 (50.0%)       | 40 (66.7%)                  | 50 (58.1%)              | —       |
| **Symptom, no. (%)** |                  |                             |                         | 0.006   |
| Headache             | —                | 34 (56.7%)                  | 33 (38.4%)              | —       |
| Visual field defect   | —                | 4 (6.7%)                    | 20 (23.3%)              | —       |
| Visual deficit       | —                | 27 (45.0%)                  | 62 (72.1%)              | —       |
| Nausea and vomiting  | —                | 6 (10.0%)                   | 11 (12.8%)              | —       |
| Polyclia             | —                | 4 (6.7%)                    | 1 (1.3%)                | —       |
| None                 | —                | 7 (8.1%)                    | 7 (11.7%)               | —       |
| **SBP (mmHg)**       | 124.70 ± 3.18    | 126.83 ± 2.00               | 124.38 ± 1.74           | 0.639   |
| **DBP (mmHg)**       | 73.10 ± 1.57     | 77.03 ± 1.16                | 76.86 ± 1.06            | 0.216   |
| **BMI (kg/m²)**      | 22.70 ± 0.48     | 23.83 ± 0.40                | 23.39 ± 0.31            | 0.222   |
| **Tumor size (mm)**  | —                | 17.90 ± 0.69                | 29.71 ± 0.86³<0.001    | —       |
| **WBC (×10⁹/L)**     | 6.62 ± 0.17      | 6.30 ± 0.19                 | 5.63 ± 0.17³<0.003     | 0.003   |
| **PRL (µg/L)**       | 10.24 ± 0.86     | 19.98 ± 2.35⁴<0.003         | 24.89 ± 1.77³<0.003    | 0.003   |
| **GH (µg/L)**        | 0.81 ± 0.23      | 0.71 ± 0.15                 | 0.59 ± 0.09             | 0.597   |
| **LH (IU/L)**        | 20.11 ± 4.91     | 6.67 ± 0.98²<0.001          | 5.52 ± 0.70⁵<0.001     | <0.001  |
| **FSH (IU/L)**       | 39.24 ± 9.90     | 15.13 ± 2.62²<0.001         | 15.24 ± 2.03²<0.001    | <0.001  |
| **E₂ (pg/ml)**       | 14.17 ± 2.92     | 22.02 ± 2.22                | 17.33 ± 1.55            | 0.094   |
| **T (ng/ml)**        | 2.64 ± 0.77      | 2.16 ± 0.31                 | 1.07 ± 0.15³<0.001     | 0.001   |
| **AÇTH (pg/ml)**     | 25.61 ± 2.27     | 26.39 ± 2.26                | 23.73 ± 1.35³<0.001    | 0.527   |
| **F (nmol/L)**       | 295.01 ± 340.59  | 342.95 ± 314.37             | 312.96 ± 301.74         | 0.641   |
| **TSH (mIU/L)**      | 19.59            | 21.77                       | —                       |         |
| **FT₄ (µg/ml)**      | 2.61 ± 0.44      | 1.53 ± 0.17²<0.003          | 1.75 ± 0.15³<0.005     | 0.015   |
| **fT₄ (ng/ml)**      | 1.18 ± 0.05      | 1.42 ± 0.15                 | 1.36 ± 0.11             | 0.644   |

Data are presented as mean ± SEM or number (percent). *P < 0.01 vs. noninvasive NFPAs, †P<0.01 vs. control, ‡P<0.05 vs. control. Continuous variables except tumor size were compared using One-way ANOVA followed by LSD. Tumor size was compared using Mann-Whitney U tests. Categorical variables were compared using Chi-square tests. Abbreviations: ACTH, adrenocorticotropin; BMI, body mass index; DBP, diastolic blood pressure; E₂, estradiol; F, cortisol; FSH, follicle-stimulating hormone; FT₄, free thyroxine; fT₄, free thyroxine; GH, growth hormone; NFPAs, nonfunctioning pituitary adenomas; PRL, prolactin; SBP, systolic blood pressure; T, testosterone; TSH, thyroid-stimulating hormone; WBC, white blood cell.
CD3⁻CD56⁺ NK Cells Were Decreased and CD3⁺CD8⁻CD28⁻ T Cells Were Increased in Peripheral Blood of Patients With Invasive NFPAs

The percentage of peripheral CD3⁻CD56⁺ NK cells and CD3⁺CD8⁻CD28⁻ T cells are shown in Figure 2. It is clear from the figures that patients with invasive NFPAs had significantly lower percentage of CD3⁻CD56⁺ NK cells, but higher percentage of CD8⁻ Tregs than patients with noninvasive NFPAs and healthy controls, but patients with noninvasive NFPAs showed no significant differences in CD3⁻CD56⁺ NK cells and CD8⁻ Tregs compared with healthy controls. In addition, neither CD3⁻CD56⁺ NK cells nor CD8⁻ Tregs were correlated with tumor size (r = −0.321, p = 0.365 and r = 0.377, p = 0.184, respectively).

Lower Infiltration of CD56⁺ Cells and CD28⁺ Cells but Greater Expression of IL-10 in Tumor Tissues of Patients With Invasive NFPAs

To further explore the role of immune tolerance or escape in IPAs, infiltration of lymphocytes and expression of inflammatory cytokines in pituitary adenomas specimens were analyzed. As shown in Figure 4B, the infiltration of CD3⁺, CD4⁺, and CD8⁺ cells were similar in patients with invasive NFPAs and noninvasive NFPAs. Moreover, Figure 4B showed that patients with invasive NFPAs exhibited significantly lower infiltration of CD56⁺ cells and CD28⁺ cells than patients with noninvasive NFPAs. IL-10 is an immunosuppressive factor, whereas both IL-2 and IFN-γ are proinflammatory cytokines. Our results showed that patients with invasive NFPAs had significantly higher expression of IL-10, lower expression of IFN-γ and IL-2 than patients with noninvasive NFPAs, but not significantly different [Figure 4B].

DISCUSSION

As suggested in the present study, the majority of NFPAs are macroadenomas and come to clinical attention due to mass effects, such as headaches, visual field defect, visual deficit, and

FIGURE 1 | Distribution and differences of T cell subpopulations among healthy controls (n = 20), patients with noninvasive NFPAs (n = 60) and patients with invasive NFPAs (n = 86). Neither the percentage of CD3⁺CD4⁺ nor the percentage of CD3⁺CD8⁺ cells was not significantly altered among the three groups. Bars represent means ± SEMs. In-NFPAs, invasive nonfunctioning pituitary adenomas; Non-NFPAs, noninvasive nonfunctioning pituitary adenomas.

FIGURE 2 | Histogram of percentage of CD3⁻CD56⁺ NK cells and CD3⁺CD8⁺CD28⁻ T cells in healthy controls (n = 20), patients with noninvasive NFPAs (n = 60) and patients with invasive NFPAs (n = 86).

FIGURE 3 | Expression of cytokines in peripheral blood of healthy controls (n = 20), patients with noninvasive NFPAs (n = 60) and patients with invasive NFPAs (n = 86).

FIGURE 4B | Infiltration of lymphocytes and expression of cytokines in pituitary adenomas specimens of patients with invasive NFPAs (n = 86) and noninvasive NFPAs (n = 60).
hypopituitarism. The invasiveness of pituitary adenomas has been correlated to age, tumor size, sex, and tumor type [6, 20]. However, our results only showed that tumor size was related with invasiveness of NFPAs as a common type of PAs. Moreover, our results also showed that the level of WBC, which contains several important immune cells and play vital roles in immune function, was lower in patients with invasive NFPAs than patients with noninvasive NFPAs, suggesting that tumor immunity could have an important role in invasiveness of NFPAs.
T cell subsets include CD3+CD4+ T cells and CD3+CD8+ T cells. CD4+ Th cells and CD8+ cytotoxic T cells play important roles in inhibiting and impeding tumor growth and killing tumor cells (as shown in Table 2). Some studies have suggested that the presence of CD4+ and CD8+ cells were correlated with improved survival in patients with certain cancers [10, 21–23]. Effector CD8+ cytotoxic T cells have been shown to be specifically associated with favorable prognosis of patients with some malignant tumors, such as glioblastomas, ovarian cancers and pancreatic cancers [22, 24, 25]. Programmed death-ligand 1 (PD-L1), as an immunosuppressive protein, was expressed highly in tumor tissue of PA patients [26], which can induce immune evasion by desensitizing the recognition and elimination of tumor cells via CD8+ T cells [27]. In addition, Hazrati et al. [28] reported that Th1 activator adjuvants and autoantigens are successful for treatment of patients with recurrent pituitary macroadenomas after operation. Our results showed that there were no statistically significant differences in the percentage of CD3+CD8+ T cells and the infiltration of CD8+ cells between patients with noninvasive NFPAs and patients with invasive NFPAs, suggesting that CD8+ cytotoxic T cells were not associated with invasiveness of NFPAs.

As a central component of the innate immune system, NK cells control several types of tumors by inhibiting tumor cell dissemination (as shown in Table 2) [29]. However, to our best knowledge, studies of the effects of NK cells on solid tumors are limited [23, 30–33]. Ma et al showed that NK cells are infiltrated in prolactinomas and non-secreting pituitary adenomas [15]. In this study, we assessed the frequencies of peripheral CD3−CD56+ NK cells as a subtype of NK cells and showed that patients with invasive NFPAs had significantly lower percentage of CD3−CD56+ NK cells than patients with noninvasive NFPAs and healthy controls, and less tumor infiltration of CD56+ cells than patients with noninvasive NFPAs. Although it is unclear whether alterations of NK cells in invasive NFPAs are the cause or responses to the adenoma, these results still indicated that a lower percentage of NK cells and lower infiltration of NK cells were related with increased invasiveness of NFPAs. The levels of CD3−CD56+ NK cells may be a useful biomarker of invasive NFPAs.
Not all T cells are antitumor effector immune cells. For example, Tregs [34, 35], including natural CD4⁺CD25⁺ Tregs (nTregs), T helper 3 (Th3) cells, type 1 Tregs (Tr1 cells), and CD8⁺CD28⁻ cells (CD8⁺ Tregs), could induce immune tolerance by suppressing host immune responses and thus inhibit effective antitumor immune responses. CD8⁺ Tregs (as shown in Table 2) is a common subset of Tregs and induces immune tolerance of antigen-presenting cells (APC), mainly dendritic cells (DCs), through cell-cell contact. Additionally, these cells can secrete IL-10 [35], transforming growth factor (TGF)-β, IFN-γ, and chemokine C-C motif ligand 4, and directly kill CD4⁺ effector T cells and APCs. Previous studies [36–38] have shown that patients with non-small cell lung cancer (NSCLC) had higher percentage of CD8⁺ Tregs than healthy controls and that this T-cell subset is correlated with pathological stage of NSCLC. Wu et al. [39] have found similar results in ovarian cancer, demonstrating that patients with ovarian cancer had higher percentage of CD8⁺ Tregs than patients with benign ovarian tumors. To the best of our knowledge, our study is the first to demonstrate that patients with invasive NFPAs had significantly higher percentage of peripheral CD8⁺ Tregs than patients with noninvasive NFPAs and healthy controls and lower tumor infiltration of CD28⁺ cells than patients with noninvasive NFPAs. Therefore, our findings exhibited that higher level of CD8⁺ Tregs and lower infiltration of CD28⁺ cells were associated with the invasiveness of NFPAs. Furthermore, CD8⁺ Tregs may be another important biomarker for the invasion of NFPAs. Our findings also indicated that the levels of peripheral CD3⁺CD56⁺ NK cells, CD8⁺ Tregs and IL-10 may be useful biomarkers for diagnosis of invasive NFPAs.

### CONCLUSION

In conclusion, the infiltrations of immune cells and expression of inflammatory cytokine were different between noninvasive NFPAs and invasive NFPAs. Low infiltration of CD56⁺ and CD28⁺ cells, as well as high expression of IL-10 in tumor tissue, were related with increased invasiveness of NFPAs. Moreover, according to the results of lymphocytes and inflammatory cytokine in peripheral blood, our findings also indicated that the levels of peripheral CD3⁺CD56⁺ NK cells, CD8⁺ Tregs and IL-10 may be useful biomarkers for diagnosis of invasive NFPAs.

### DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/Supplementary Material.

### ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Shanghai Fifth People’s Hospital.

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**TABLE 2 | Immunological role of several immune cells and inflammatory cytokines.**

| Parameters | Immunological role |
|---|---|
| CD3⁺CD4⁺T lymphocytes and CD3⁺CD8⁺T lymphocytes | As the subsets of T cell, CD3⁺CD4⁺T lymphocytes and CD3⁺CD8⁺T lymphocytes play important roles in inhibiting and impeding tumor growth and killing tumor cells. However, neither one of them was associated with invasiveness of NFPAs in our study. |
| CD3⁺CD56⁺ NK cells | As a central component of the innate immune system, CD3⁺CD56⁺ NK cells as a subtype of NK cells control several types of tumors by inhibiting tumor cell dissemination. Our findings indicated that the reduction of NK cells was related with increased invasiveness of NFPAs and may be a useful biomarker of invasive NFPAs. |
| CD8⁺CD28⁻ cells (CD8⁺ Tregs) | As a common subset of tregs, CD8⁺ tregs that render antigen presenting cells (APC), mainly dendritic cells (DCs), tolerant through cell-cell contact, can secrete IL-10, TGF-β, IFN-γ, CCL4, and directly kill CD4⁺ effector T cells and APCs. Our findings suggested that high level of CD8⁺ tregs and low infiltration of CD28⁺ cells were associated with the invasiveness of NFPAs. CD8⁺ tregs may be another important biomarker for the invasion of NFPAs. |
| IL-10 | IL-10 is mainly produced by Th2 cells. As an immunosuppressive factor that is generally thought to support tumor growth and progression. Similarly to CD8⁺ tregs and CD56⁺ NK cells, high expression of IL-10 in tumor tissue was also related with increased invasiveness of NFPAs. IL-10 may present a useful biomarker for diagnosis of invasive NFPAs. |
| IFN-γ and IL-2 | Th1 cells can secrete IFN-γ and IL-2, which activate macrophages, NK cells, and cellular immunity, and play important role in protection against tumor cells. But neither one of them was associated with invasiveness of NFPAs in our study. |

CCL4, chemokine C-C motif ligand 4; IL-10, interleukin-10; IL-2, interleukin-2; IFN-γ, interferon-γ; TGF-β, transforming growth factor-β; NFPAs, nonfunctioning pituitary adenomas; NK, natural killer.
Hospital, Fudan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XH, JX, JL, and SZ were major contributors in writing the manuscript. JY, YL, and BZ completed the acquisition and analysis of the patient data. XY, YT, TS, and LS performed research and developed the tool. All authors reviewed and approved the final version of the manuscript.

INFORMED CONSENT

All participants gave written informed consent to participate in the study in accordance with the Declaration of Helsinki.

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