Dynamic Biomarkers Indicate Immunological Benefits Provided by Ganoderma Spore Powder in Post-Operative Breast and Lung Cancer Patients: A Prospective Clinical Trial

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Primary research

Keywords: Ganoderma spore powder, immune microenvironment, T cell subsets, cytokines, neutrophil–lymphocyte ratio, albumin-to-globulin ratio, breast cancer, lung cancer

DOI: https://doi.org/10.21203/rs.3.rs-47879/v1

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Abstract

Background: T lymphocyte subsets with correlative cytokines, albumin to globulin ratio (AGR) and neutrophil to lymphocyte ratio (NLR), have been linked to inflammatory indicators of immune response and prognosis in various cancer types. The aim of this study was to determine the utility of these biomarkers in predicting the immunological benefits of Ganoderma spore powder in post-operative patients with breast and lung cancer.

Methods: We prospectively evaluated 120 consecutive breast and lung cancer patients with or without Ganoderma spore powder. T lymphocyte subsets, as well as relative cytokines were detected and assessed by Pearson correlation analysis. The relationships between AGR and NLR with ganoderma spore powder treatment and prognosis were analyzed using Kaplan–Meier and Cox regression methods.

Results: The prevalence of CD3+, CD4+ and CD4+/CD8+, CD3+HLADR-, CD4+HLADR- and CD8+HLADR- cell types was higher in the ganderma spore treated group compared to untreated controls. In addition, the percentage of CD4+CD25+Treg, CD3+HLADR+, CD4+HLADR+ and CD8+HLADR+ cell types was lower in the treated group. IL-2 and IL-12 frequencies were significantly higher during the treatment period which negatively impacted levels of IL-10. Other immunosuppressive factors such as COX2, TGF-1 and HIF-1A had higher prevalence in non-treated patients. Correlation analysis showed several correlations between the biomarkers, specifically between IL-6 and TGF-1, CD28 and IL-12R, TGF-1 and Foxp3, IL-10 and IL-12R with COX2, Foxp3 and CD8, IL-10 and CD28 with IL-12R, COX2 and CD8, IL-12R with COX2, CD3 with CD4 and CD8. A positive relationship was also observed between IL-2 and TGF-1. Conversely, IL-2 was negatively related to CD3, and HIF-1 negatively related with CD4, CD28 and CD3. Kaplan–Meier analysis suggested that low AGR/high NLR was related to poor progression free survival (PFS, HR=1.572, P=0.038/HR=2.064, P=0.042). A combination of high AGR and low NLR that predicted treatment benefits was also associated with PFS (HR=0.661, P=0.033/HR=1.044, P=0.048).

Conclusions: Our findings show that T lymphocyte subsets predominately combined with relevant cytokines and AGR/NLR inflammatory predictors may help to identify patients most likely to benefit from the immunological enhancements from Ganoderma spore powder treatment.

Background

Lung cancer is the leading cause of cancer death worldwide. Approximately 1.8 million new people were diagnosed during 2012 which accounted for 1.6 million deaths [1]. Breast cancer is the leading cause of cancer death in women accounting for 1.2 million cases and around 500,000 deaths worldwide [2]. Conventional treatments with surgery or chemo-radiotherapy inevitably result in patients being immunocompromised during treatment [3] which can significantly impact outcomes. Currently, there is a critical unmet clinical need for the development of novel biomarkers to better identify patients likely to respond to immune-modulatory drugs.

Traditional Chinese medicines hold the theory of "the same origin of medicine and food" which have been reported have pronounced effects on regulation of the immune system and anti-tumor effects [4–5]. Specifically, G. lucidum polysaccharides have been shown to affect T/B lymphocytes, macrophages, dendritic and natural killer cells. Studies have indicated that the water-soluble polysaccharide (GSG) component of G. lucidum spores can elevate IL-6 and TNF-α levels in murine resident peritoneal macrophages partially through the glucan receptor, Dectin-1, and MAPKs- and a Syk-dependent pathway [6–8]. It has also be shown that G. lucidum can also induce the expression of IL-2, IL-4 and IL-10 in murine models of gastric cancer [9–10]. Similarly, the effects of triterpenes have been shown to mediate MAP kinases p38 and JNK rather than NF-κB activation which could act to enhance the immunological functions of monocytes [9].
T lymphocytes are the main effector cells which drive anti-tumor immunity. Successful anti-tumor immunity requires coordination and cooperation of various T cell subtypes including helper CD4+ cells and cytotoxic CD8+ cells [11–12]. Recently, inflammation-associated biomarkers have attracted major attention as predictive tools in cancers including parameters such as neutrophil–lymphocyte ratio (NLR) and albumin-to-globulin ratio (AGR) [13–14]. In this study, we aimed to determine the impact of *G. lucidum* spore treatment on key immunological biomarkers including T cell sub-populations, cytokine levels, AGR and NLR in lung and breast cancer patients.

**Methods:**

**Design.** This study was designed as a randomized controlled trial to compare the *G. lucidum* treated group with untreated patients. In the treatment group, patients were treated with 2000 mg of *G. lucidum* spore powder three times a day for 6 weeks (obtained from the Jiangxi Tianhai Group, Batch number 2014AA022206-LC02). Placebo was applied in untreated group for 6 weeks. At the end of the treatment period, CD3+, CD4+ T cell subsets and inflammatory factors such as Foxp3+ and TGF-β were detected. Routine laboratory data (neutrophil and lymphocyte absolute count, serum ALB and GLB value) were collected during follow-up. PFS was calculated from the first cycle of treatment to the time of documented tumor progression or death.

**Participants.** The study protocol and all procedures were approved by The Institutional Review Board of the Third Affiliated Hospital of Harbin Medical University. All participants were recruited to the study after signed informed consent and were receiving post-operative adjuvant chemotherapy for breast and lung cancer at the Third Affiliated Hospital of Harbin Medical University from June 2015 to September, 2016. All patients eligible for the study who met the inclusion criteria were assessed by periodic imaging, hematology examination including immune function (on the 21st and 42nd day) and biochemical indices. Outcomes including PFS were determined by clinical evidence.

The eligibility criteria for recruitment to the study were as follows (a) provision of signed informed consent; (b) >18 years of age; (c) diagnosed with stage I-IIIA breast cancer and non-small cell lung cancer (NSCLC); (d) ECOG score between 0 and 1; (e) Estimated survival period >3 months; (f) no other underlying diseases leading to immune-compromise; (g) previous surgical treatment and undergoing standard adjuvant chemotherapy. The exclusion criteria were as follows (a) allergic to the *G. lucidum*; (b) Uncontrolled and symptomatic brain metastases; (c) abnormal levels of serum alanine transaminase (ALT), aspartic acid transaminase (AST), blood urea nitrogen (BUN), or creatinine; (d) hematological disease with hemoglobin levels <9 g/dL and platelets <80000/mL.

**Immunological Indicators:**

**Flow cytometry analysis.** Forearm venous blood samples were separately collected in 5 mL vacutainers containing EDTA and unfractionated heparin. Flow cytometry was performed according to the manufacturer’s instructions. A BD LSRII flow cytometer (BD Biosciences, Heidelberg, Germany) was used to flow cytometry analysis. The TSNE algorithm from FlowJo software (FlowJo, Ashland, OR, USA) was used to dimensional reduce cell clustering. The following fluorescent antibodies were used for analysis; anti-CD3 PerCP(SK7), anti-CD8 FITC-(SK1), anti-CD25 APC (2A3), anti-CD4 V500(RPAT4), anti-HLA-DR PerCP-Cy5.5 (L243) (obtained from Becton-Dickinson, San Diego, CA, USA), Anti-CD28-APC(CD28.2) (obtained from eBioscience, San Diego, CA) and CD16-FITC, CD56-PE, CD29-FITC, CD45RA-FITC, CD45RO-PE, CD19-FITC (obtained from Beckman-Coulter, USA). T-cell subtypes were differentiated following phenotypic analysis (Fig. 1). This included identification of the following cell subtypes; Total T lymphocytes (CD3+), helper T cells (CD4 + CD8−), cytotoxic T cells (CD4− CD8 + CD28+), inhibitory T cells (CD4-CD8 + CD28-) and Tregs (CD4 + CD25 high). Expression of the late activation marker, HLA-DR, was determined. Naive (CD45RA + CD45RO−) and memory T cells (CD45RA-CD45RO+) were also distinguished in CD4 + and CD8 + subsets.
RNA isolation and RTqPCR analysis. Total RNA was extracted by TRIzol reagent according to the manufacturer’s protocol. The PrimeScript RT Reagent Kit (Takara) was used to synthesize cDNA. QPCR was conducted using a SYBR Green Master mix (Roche) three times. A standard curve method was used to evaluate the relative expression of genes and β-Actin designated as an internal expression reference gene. The gene-specific PCR primers are summarized in Supplementary Table 1. Of these primers, TGF-1 and COX-2 demonstrated negative associations with tumor immunity. HIF-1 was associated with the hypoxic response. IL-12R demonstrated consistent association with Th1 cells. IL-2 demonstrated positive associations with Th1 cells, whilst IL-6, IL-10 and TGF-1 were negatively associated with Th2 cells [15].

Clinical data collection. Laboratory data was periodically collected including neutrophil/lymphocyte absolute counts and serum ALB/ GLB. NLR values were defined by dividing the absolute neutrophil count by the absolute lymphocyte count. AGR was reported as the ALB value divided by the GLB value.

Safety and Toxicity. Supervised safety and toxicity was reported for renal (sodium, potassium and urea creatinine) and liver function (total protein, albumin, total bilirubin, alkaline phosphate and alanine transaminase) during follow-up. Adverse effects were assessed using the NCICTC, version 4.0 scale. The Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1) was used to evaluate cancer progression.

Data analysis

All data analyses were performed by clinical oncologists based on descriptive statistical analysis of demographic variables that were used to assess the clinical characteristics of the study samples. The data of T cell subsets was analyzed using FlowJo software (version 7.6.1, FlowJoLLC, Ashland, OR, USA). Differences in T-cell subsets and cytokines in the same patients were statistically compared using a paired t test. The Spearman's test was conducted to determine correlation between cytokine genes and T cell-associated markers. The impact of Ganoderma spore powder on AGR and NLR with PFS was analyzed using the Kaplan–Meier method with a two-tailed Log-rank test to determine statistical significance. The Cox proportional hazard model for the uni- and multivariate analysis was established to assess the prognostic variables based on PFS. Comparison of P < 0.05 (bilateral) was statistically significant. Data statistics were analyzed by SPSS, version 22 and GraphPad Prism, version 7.

Results:

Baseline characteristics of participants. From June 2015 to September 2016, 120 NSCLC and breast cancer patients undergoing postoperative adjuvant chemotherapy were randomized into treatment (63) or a non-treated (57) groups. Although *G. lucidum* has previously been shown to be well tolerated, there have been cases reporting that intake of GLSP may effect CA72-4 expression causing abnormal elevation [16]. Li et al., demonstrated correlation between the biological activity and cytotoxicity of ganoderma spore powder with a recommended effective dose of 4 g [17–18]. No significant differences in baseline characteristics between the two groups were determined from the clinical data summarized in Table 1. The distribution of treatments was not significant in the treated group (Pearson chi-square P = 0.34), although a slightly larger proportion of patients had previously been treated with three cytotoxic therapies compared to one, due to adjuvant chemotherapy.
Table 1
Demographics of patients in each group.

|                               | Drug group (n = 63) | Control group (n = 57) | P-value |
|-------------------------------|---------------------|------------------------|---------|
| (1) age (year) mean (SD)      | 57.6(37–87)         | 55.8(38–92)            | P > 0.1 |
| (2) Body mass index (kg/m2) mean (SD) | 25.2 (18.6–51.3) | 23.5 (17.1–40.5) | P > 0.1 |
| (3) Clinical stage n (%)      |                     |                        |         |
| Stage I                       | 10 (15.9%)          | 8 (15.1%)              | P > 0.05|
| Stage II                      | 36 (57.1%)          | 34 (59.6%)             |         |
| Stage IIIA                    | 17 (27.0%)          | 15 (26.3%)             |         |
| (4) pathological type n(%)    |                     |                        |         |
| NSCLC                         | 27 (42.9%)          | 24 (42.1%)             | P > 0.05|
| Breast cancer                 | 36 (57.1%)          | 33 (57.9%)             |         |
| (5) ECOG performance status score |                 |                        |         |
| 0                             | 45 (71.4%)          | 42 (73.7%)             | P > 0.05|
| 1                             | 17 (27.0%)          | 14 (24.6%)             |         |
| 2                             | 1 (1.6%)            | 1 (1.8%)               |         |
| (6) No. of prior cytotoxic regimens |            |                        |         |
| 1                             | 15 (23.8%)          | 12 (21.1%)             | P > 0.05|
| 2                             | 0                   | 0                      |         |
| 3                             | 0                   | 0                      |         |

Statistical Analysis Of T Cell Subsets

Of all enrolled patients, 63 patients who received drug showed an induction of CD4 + and CD8 + T cells restricted by HLA molecules including HLADR. A significant increase in proliferation of the HLADR- subset was observed in the treated group, whilst 57 patients had pre-existing responses that were boosted in the HLADR + subset. NKT and Treg cells showed lower immune responses after adjuvant therapy. Antitumor CTL was decreased compared to the treated group, but no statistically significant differences were found (P > 0.05). Furthermore, the associated induction of CD3 + T, CD4 + CD25 + T and other cells before and after therapy was considered to be less different. The bias compared the T cell response with the function of ganoderma spore powder was tested mostly owing to drug bioactivity [19], as depicted in Table 2 and Fig. 1.
Table 2
Detection results of T cell subsets in treated and untreated groups.

| T cell subset(%) | untreated group(N = 57) | treated group(N = 63) | P    |
|------------------|--------------------------|------------------------|------|
| CD3+             | 66.4 ± 10.6              | 72.0 ± 6.0**           | 0.001|
| CD3+CD4+         | 37.7 ± 10.5              | 42.0 ± 6.4*            | 0.010|
| CD3+CD8+         | 26.1 ± 7.9               | 26.1 ± 6.4             | 0.978|
| CD4+/CD8+        | 1.5 ± 0.8                | 1.8 ± 0.7*             | 0.087|
| CD3-CD16 + CD56+ | 8.2 ± 6.6                | 6.7 ± 4.5              | 0.232|
| CD3 + CD16 + CD56+ | 16.9 ± 11.0          | 12.5 ± 6.0**           | 0.007|
| CD4 + CD29+      | 18.4 ± 7.0               | 20.2 ± 4.8             | 0.126|
| CD4 + CD25+      | 10.0 ± 4.0               | 8.4 ± 3.5*             | 0.046|
| CD3 + HLA DR+    | 9.7 ± 6.5                | 1.7 ± 1.0**            | 0.000|
| CD3 + HLA DR-    | 56.3 ± 12.5              | 70.4 ± 5.6**           | 0.000|
| CD4 + HLA DR+    | 3.5 ± 2.4                | 1.9 ± 1.0**            | 0.000|
| CD4 + HLA DR-    | 37.0 ± 10.8              | 41.9 ± 6.8**           | 0.004|
| CD8 + HLA DR+    | 5.3 ± 5.0                | 0.7 ± 0.5**            | 0.000|
| CD8 + HLA DR-    | 24.9 ± 8.0               | 28.2 ± 6.8*            | 0.040|
| CD4 + CD45RA+    | 14.6 ± 8.2               | 16.3 ± 7.6             | 0.304|
| CD4 + CD45RO+    | 27.5 ± 8.3               | 27.8 ± 5.8             | 0.852|
| CD8 + CD28+      | 14.1 ± 4.8               | 14.7 ± 4.9             | 0.527|
| CD8 + CD28-      | 16.9 ± 8.3               | 15.2 ± 6.7             | 0.285|

Note: compared with those in the untreated group, *P < 0.05 and **P < 0.01, respectively.

Table 3
the expression of objective cytokine gene in patients with or without ganoderma spore powder.

| GENES    | untreated group χ²±s | treated group χ²±s | F values | t    | P    |
|----------|----------------------|---------------------|----------|------|------|
| IL-10    | 4.61 ± 1.75          | 3.81                | 1.07 ± 0.62  | 5.945 | 0.000|
| IL-2     | 1.48 ± 1.32          | 2.15                | 4.09 ± 1.55  | 5.697 | 0.000|
| IL-12    | 2.28 ± 1.94          | 3.83                | 7.00 ± 3.54  | 4.525 | 0.000|
| TGF-β1   | 2.13 ± 1.73          | 1.57                | 1.19 ± 1.05  | 8.555 | 0.000|
| HIF-1α   | 0.34 ± 1.94          | -2.36               | 0.49 ± 0.15  | 3.589 | 0.001|
| COX2     | 0.42 ± 0.74          | 0.12                | 1.50 ± 0.68  | 7.572 | 0.000|
Cytokines Associated With Response To Ganoderma Spore Powder

Due to the association of cytokine gene expression with T cell activity, the mRNA expression profiles of inflammatory mediators determined [20]. For patients who received adjuvant therapy, statistically significant increases in serum levels of COX2, IL-10, TGF-1, HIF-1A level were observed in the treated compared to the non-treated subjects (P < 0.001). In contrast, IL-12 and IL-2 were predominantly higher without drug interference (P < 0.001). Foxp3 and IL-6 levels showed no significant difference between the treated and non-treated groups (data not shown).

Exploratory analysis of T cell subset associations with cytokine release

Given the complex interactions of the immune system, evaluation of a single cell or cytokine is insufficient to accurately assess the overall state of the immune microenvironment [21]. Spearman correlation analyses was performed to control meaningful variables. The levels of several cytokines were shown to negatively correlate with T cell activity, namely, IL-2 and CD3, HIF-1 and CD4, CD28 and CD3. Statistical analysis of the effect of ganoderma spore powder as a primary covariate and regression analysis suggested that a positive correlation trend across T cell phenotype with statistical significance for IL-6 with TGF-1, CD28 and IL-12R, TGF-1 with IL-2, Foxp3, IL-10, IL-12R and COX2, Foxp3 with CD8, IL-10 with CD28 and IL-12R, COX2 with CD8, IL-12R with COX2, CD3 with CD4 and CD8 (Table 4). These analyses demonstrated that activated molecules are differentially associated with cytokine production following drug stimuli suggesting a high possibility for effective evaluation of overall immune status.
Table 4
Correlation analysis among immune modulators

|        | IL-6 | IL-2 | TGF-β | Foxp3 | HIF-1α | IL-10 | CD28 | IL-12R | CD3 | CD4 | CD8 |
|--------|------|------|-------|-------|--------|-------|------|--------|-----|-----|-----|
| IL-2   | CC#  | 0.08 |       |       |        |       |      |        |     |     |     |
|        | Sig# | 0.49 |       |       |        |       |      |        |     |     |     |
| TGF-β1 | CC#  | 0.25**| 0.39**|       |        |       |      |        |     |     |     |
|        | Sig# | 0.01 | 0.00  |       |        |       |      |        |     |     |     |
| Foxp3  | CC#  | -0.14| -0.01 | 0.21  |        |       |      |        |     |     |     |
|        | Sig# | 0.20 | 0.96  | 0.05* | 0.00   |       |      |        |     |     |     |
| HIF-1α | CC#  | 0.07 | 0.02  | 0.03  | -0.17  |       |      |        |     |     |     |
|        | Sig# | 0.47 | 0.88  | 0.77  | 0.15   |       |      |        |     |     |     |
| IL-10  | CC#  | 0.11 | -0.34 | 0.22* | -0.02  | -0.05 |      |        |     |     |     |
|        | Sig# | 0.30 | 0.05* | 0.03  | 0.86   | 0.62  |      |        |     |     |     |
| CD28   | CC#  | -.23*| 0.18  | -.21* | 0.08   | -0.13 | -0.23*|        |     |     |     |
|        | Sig# | 0.02 | 0.10  | 0.04  | 0.47   | 0.19  | 0.03  |        |     |     |     |
| IL-12R | CC#  | 0.25*| 0.11  | .37** | 0.16   | 0.11  | .30** | 0.18   |     |     |     |
|        | Sig# | 0.01 | 0.34  | 0.00  | 0.12   | 0.28  | 0.00  | 0.07   |     |     |     |
| CD3    | CC#  | 0.01 | -.29**| 0.02  | -0.13  | 0.09  | 0.03  | -0.28**| 0.03|     |     |
|        | Sig# | 0.90 | 0.01  | 0.82  | 0.21   | 0.40  | 0.81  | 0.00   | 0.77|     |     |
| CD4    | CC#  | -0.01| -0.08 | -0.11 | -0.09  | -.20**| 0.05  | -0.10  | -0.01| .35**|     |
|        | Sig# | 0.90 | 0.46  | 0.27  | 0.40   | 0.05  | 0.63  | 0.32   | 0.95| 0.00|     |
| CD8    | CC#  | -0.01| 0.05  | 0.00  | -0.01  | -0.02 | 0.06  | -0.11  | -0.07| 0.10| .22*|
|        | Sig# | 0.93 | 0.65  | 0.99  | 0.95   | 0.05  | 0.56  | 0.27   | 0.47| 0.31| 0.02|
| COX2   | CC#  | 0.12 | 0.02  | .33** | -0.01  | 0.09  | .27** | 0.13   | .28**| 0.03| 0.04|
|        | Sig# | 0.20 | 0.84  | 0.00  | 0.96   | 0.44  | 0.01  | 0.20   | 0.01| 0.77| 0.66|

* P < 0.05, ** P < 0.01
# CC correlation coefficient, Sig significance

Univariate and multivariate COX regression analysis of AGR and NLR

During follow-up, 113 patients (94.2%) had no disease progression, whilst 6 patients (3.8%) had progressive disease and late death. Similar to the previous studies [13, 22], Kaplan-Meier univariate survival analysis of AGR and NLR detection in each group showed that AGR, as well as PFS were positively correlated amongst breast cancer and lung cancer patients (HR = 1.572, P = 0.038). NLR was shown to be negatively correlated with PFS (HR = 2.064, P = 0.042), as
shown in Fig. 3 and Table 5. We further included ganoderma spore powder as an influencing factor for COX multivariate risk analysis. The results demonstrated that the increase of AGR was positively correlated with PFS (HR = 0.661, P = 0.033) and the decrease of NLR was positively correlated with PFS (HR = 1.044, P = 0.048), as shown in Table 5. These data indicated that ganoderma spore powder had a positive impact on patient prognosis.

| Univariate analysis | Multivariate analysis |
|---------------------|-----------------------|
| Variable            | HR(95% CI)             | P-value | HR(95% CI) | P-value |
| AGR                 | 1.572                 | 0.038*  | 0.661      | 0.033*  |
| NLR                 | 2.064                 | 0.042*  | 1.044      | 0.048*  |

* P < 0.05

**Safety And Toxicity**

With the exception of slight discomfort, no serious adverse effects were observed reported as summarized in Table 6. The data showed that dizziness (16.0%) and feeble (12.0%) were dominating symptoms. Renal (sodium, potassium, and urea creatinine) and liver function tests (total protein, albumin, total bilirubin, and alanine transaminase) showed no abnormal major fluctuations. These indices were within the normal range of the measurements during study unless otherwise stated.
### Table 6
Mild adverse events of patients in Experimental versus Control Group.

| System        | Event                          | Maximum Grade | Experimental group (n = 63) | Control group (n = 57) | x² P  |
|---------------|--------------------------------|---------------|--------------------------|------------------------|-------|
| General       | Fatigue                        |               | 2(4) 0(0) 0(0)           | 21(42) 2(4) 0(0)       | < 0.05|
|               | Dizzy                          |               | 3(6) 0(0) 0(0)           | 5(10) 3(6) 0(0)        | 0.09  |
| GI            | Colitis or diarrhea            |               | 0(0) 0(0) 0(0)           | 1(1) 0(0) 0(0)         | 1.00  |
|               | Nausea with or without emesis  |               | 0(0) 0(0) 0(0)           | 1(0) 3(6) 0(0)         | 1.00  |
|               | Pancreatitis                   |               | 0(0) 0(0) 0(0)           | 0(0) 0(0) 0(0)         | 1.00  |
|               | Liver enzyme elevation         |               | 1(2) 0(0) 0(0)           | 2(4) 0(0) 0(0)         | 0.50  |
|               | Pancreatic enzyme elevation    |               | 0(0) 0(0) 0(0)           | 0(0) 0(0) 0(0)         | 1.00  |
| Hematologic   | Anemia with or without hemolysis|               | 1(2) 0(0) 0(0)           | 3(6) 1(2) 0(0)         | 0.24  |
|               | Neutropenia                    |               | 2(4) 0(0) 0(0)           | 6(12) 5(10) 1(2)       | 0.15  |
|               | Thrombocytopenia               |               | 0(0) 0(0) 0(0)           | 1(2) 0(0) 2(4)         | 0.36  |
| Dermatologic  | Rash                           |               | 0(0) 0(0) 0(0)           | 0(0) 2(4) 0(0)         | 0.50  |
| Endocrine     | Hypothyroidism                 |               | 0(0) 0(0) 0(0)           | 2(4) 0(0) 0(0)         | 1.00  |
|               | Hyperthyroidism                |               | 0(0) 0(0) 0(0)           | 0(0) 0(0) 0(0)         | 1.00  |
|               | Adrenal insufficiency          |               | 0(0) 0(0) 0(0)           | 0(0) 0(0) 0(0)         | 1.00  |
|               | Hyperglycemia                  |               | 0(0) 0(0) 0(0)           | 1(2) 0(0) 0(0)         | 0.50  |
|               | Other                          |               | 0(0) 0(0) 0(0)           | 0(0) 0(0) 0(0)         | 1.00  |
| Musculoskeletal| Arthritis or arthralgia        |               | 0(0) 0(0) 0(0)           | 1(2) 0(0) 0(0)         | 0.05  |
| Respiratory   | Dyspnea                        |               | 1(2) 0(0) 0(0)           | 3(6) 1(2) 0(0)         | 0.56  |
|               | Pneumonitis                    |               | 0(0) 0(0) 0(0)           | 1(2) 0(0) 0(0)         | 0.50  |
|               | Hypoxia                        |               | 2(4) 0(0) 0(0)           | 1(2) 3(6) 0(0)         | 0.71  |
| Renal         | Acute kidney injury            |               | 0(0) 0(0) 0(0)           | 1(2) 1(2) 0(0)         | 1.00  |
| Neurologic    | Encephalopathy                 |               | 0(0) 0(0) 0(0)           | 1(2) 0(0) 0(0)         | 1.00  |

Supplementary Table 1. Primers used for real-time quantitative PCR

**Discussion**
This pilot study is the first to reveal the impact of an individualized traditional Chinese medicine intervention on the immune system in breast and lung cancer patients during adjuvant chemotherapy. Our results showed a powerful correlation between T cell activation with inflammatory cytokines, AGR, NLR and ganoderma spore powder treatment. Our previous study determined that inhibition of NF-κB activation in immune cells induced by treatment was attributable to the active components of ganoderma triterpenes (e.g. ganoderma acid H). These effects were due to NF-κB entering the nucleus which perturbed the transcription of downstream signal molecules and improved fatigue amongst breast cancer patients receiving endocrine therapy [23]. A distinct lack of biomarkers beyond the current standard of inflammatory indices has limited the ability to select patients most likely to benefit from ganoderma spore powder treatment. T cell profiling is a promising biomarker approach where we showed that CD3+, CD4+ and CD4+/CD8+ levels were significantly higher for patients who experienced drug response, in contrast to single CD8+ subsets.

Similarly, T cell dysfunction occurs owing to complex micro environmental alterations [24]. We considered that ganoderma spore powder may exert antitumor immunity by modulation of the TME. Our laboratory and others have demonstrated that immunosuppressive CD8+CD28- levels are slightly higher in non-treated compared to treated patients [25–26] with inhibited T cell levels indicated associated with drug response, although these changes remained statistically insignificant.

Treg cell (CD4+CD25+) activation was enhanced in control subjects (P < 0.05). Treg cells have been shown to inhibit cytotoxic CD8+ T cells in various cancers. Li et al. revealed that the CD8+/Treg value is considered as a more robust relationship in prognosis with lacking evidence of increasing Treg cell in ovarian cancer [27]. Although CD8+/Treg values are currently infeasible as evidence for independent immune therapy decision-making, high intra-tumoral heterogeneity of the immune microenvironment may be dependent on more comprehensive indices to predict immune signatures. HLA-DR, as a T lymphocyte immune-regulatory molecule has been shown to vary during the cancer progression [28]. Saraiva et al. demonstrated that HLA-DR expression significantly declined in cancer subjects and is strongly connected with prognosis [29–30], as reported in 67% of gastric cancer cases and in 80% of poorly differentiated adenocarcinoma [31]. Intriguingly, our study showed that HLA-DR co-expression in CD3, CD4 and CD8 subsets was a significant trend towards suppression of drug stimuli. However, our study involved relatively small numbers and our preliminary analysis should be considered exploratory in the absence of later stage trials.

In addition to the observed effect on lymphocyte immuno-phenotype, the active components of ganoderma spore powder inhibited the expression of iNOS and COX-2, as well as TNF-α and IL-6, through the suppression of LPS-based activation of NF-κB in RAW264.7 cells [32–33]. Similarly, we presented very interesting data on Th1/Th2 cell drift. It is also noteworthy that patients treated drug compared to those who received only basal treatment showed a robust elevation in IL-2 and IL-12 as well as other immune-suppressive molecules including COX2, TGF-β1 and HIF-1a. In addition, a significant decline in IL-10 was also observed.

Che et al., showed that COX2 could sustain prostaglandin E2 (PGE2) and also boost IL-10. IL-12 exerted decreased activation in this early adjusting microenvironment setting [34]. In our study, anti-inflammatory factor Th2 drift along with COX2, TGF-β1 and HIF-1a levels which impacted the pro-inflammatory factor Th1, were regulated by ganoderma spore powder. The effects may partially lead to recovery of post-resuscitation immune dysfunction [35–36]. Taken together, the imbalance of Th1/Th2 had the ability to predict response.

Change between the levels of immune cells and inflammatory cytokines was not always consistent in TME [37–38]. Until a consensus is reached, correlation of these factors must be assessed against clinical outcomes [39]. Correlational analysis showed that TGF-β1, CD28 and IL-12R demonstrated a higher peak level of the pro-inflammatory IL-6. TGF-1 was positively correlated with IL-2, Foxp3, IL-10, IL-12R and COX2 that was represented by the samples in
this study. Alterations in local cytokine biology, particularly imbalances between pro- and anti-inflammatory cytokines, may induce many other changes associated with T cell activation, such as Foxp3 with CD8, IL-10 with CD28 and COX2 with CD8. Therefore, distinct relationships between cytokine profile and T cell phenotype could potentially make important contributions towards the effective evaluation of immunological remodeling.

Recently, there has been growing interest from Wang et al. in correlating AGR and NLR amongst the host immune system [40–41]. TNF and IL-6 as inflammatory products could reduce ALB synthesis in liver cells. Study have revealed that decreasing ALB levels represent poor prognosis in various cancers. GLB was deprived from immune organ to indicate immune function including acute reactive protein. Neutrophils could inhibit cytotoxic lymphocytes through the release of arginase, reactive oxygen species and nitric oxide. Lymphocyte counts indicate anti-tumor immunity [13]. A previous study confirmed that ganoderma spore powder inhibits the production of TNF-α and IL-6 preventing sustained immune response [32]. Against these data, surprisingly, we showed by multivariate analysis that low AGR was an independent poor prognostic factor related to PFS in the efficacy of ganoderma spore powder (HR = 1.572, p = 0.038). In contrast, high NLR levels were found to exhibit superior prognostic value (HR = 2.064, P = 0.042). We hypothesize that changes in AGR and NLR were are useful prognostic indicators based on immunological improvement, although other risk factors such as dehydration or fluid retention may cause a skewed distribution in a the small study size.

Conclusions

Our findings strongly suggested that the combination of T lymphocyte subsets relevant cytokines might offer promising strategies to identify immunological benefits in patients responding to ganoderma spore powder. AGR and NLR may be used as appropriate prognostic tools.

Declarations

Ethics approval and consent to participate

This study, including the procedures for patient enrollment and recruitment, was approved by the Institutional Review Board of the Harbin Medical University Cancer Hospital, and all patients who participated in the study provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Consent for publication

This is not applicable for this article.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Funding

Not applicable.

Acknowledgments

The ganoderma spore powder was supplied by Jiangxi Tianhai Group(Batch number 2014AA022206-LC02). The clinical data detection was supported by Harbin Medical University Cancer Hospital.

Authors’ contributions
Qingyuan Zhang designed and directed this study. Dabei Tang and Jianli Ma directed the statistical analysis of the data. Yuwei Deng wrote and polished the manuscript. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

**Competing interests**

The authors report no conflicts of interest in this work.

**References**

1. Wiel C, Le Gal K, Ibrahim MX, et al. *BACH1 Stabilization by Antioxidants Stimulates Lung Cancer Metastasis*. Cell. 2019 Jul 11;178(2):330–345.e22.

2. Sachs N, de Ligt J, Kopper O, et al. *A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity*. Cell. 2018 Jan 11;172(1–2):373–386.

3. Matikas A, Margolin S, Hellström M, et al. Long-term safety and survival outcomes from the Scandinavian Breast Group 2004-1 randomized phase II trial of tailored dose-dense adjuvant chemotherapy for early breast cancer. Breast Cancer Res Treat. 2018 Apr;168(2):349–55.

4. Xu J, Li P. Researches and Application of Ganoderma Spores Powder. Adv Exp Med Biol. 2019;1181:157–86.

5. Su J, Li D, Chen Q, et al. Anti-breast Cancer Enhancement of a Polysaccharide From Spore of Ganoderma lucidum With Paclitaxel: Suppression on Tumor Metabolism With Gut Microbiota Reshaping. Front Microbiol. 2018 Dec 17;9:3099.

6. Zhu LF, Yao ZC, Ahmad Z, Li JS, Chang MW. Synthesis and Evaluation of Herbal Chitosan from Ganoderma lucidum Spore Powder for Biomedical Applications. Sci Rep. 2018;8:14608.

7. Sliva D, Sedlak M, Slivova V, et al. Biologic activity of spores and dried powder from Ganoderma lucidum for the inhibition of highly invasive human breast and prostate cancer cells. J Altern Complement Med. 2003;9:491–7.

8. Dudhgaonkar S, Thyagarajan A, Sliva D. Suppression of the inflammatory response by triterpenes isolated from the mushroom Ganoderma lucidum. Int Immunopharmacol. 2009;9(11):1272–80.

9. Cheng S, Sliva D. Ganoderma lucidum for cancer treatment: we are close but still not there. Integr Cancer Ther. 2015 May;14(3):249–57.

10. Pan Y, Zhao A, Zhong Z, et al. Ganoderma spore lipid protects mouse bone marrow mesenchymal stem cells and hematopoiesis from the cytotoxicity of the chemotherapeutic agent. Biotechnol Prog. 2019 Sep;35(5):e2869.

11. Hargadon KM, Johnson CE, Williams CJ. Immune checkpoint blockade therapy for cancer: An overview of FDA-approved immune checkpoint inhibitors. Int Immunopharmacol. 2018 Sep;62:29–39.

12. Ma QY, Chen J, Zhao J. Follicular cytotoxic CD8 T cells present high cytokine expression, and are more susceptible to Breg-mediated suppression in non-small cell lung cancer. Immunol Res. 2020 Feb;68(1):54–62.

13. Xuan Q, Yang Y, Ji H, et al. Combination of the preoperative albumin to globulin ratio and neutrophil to lymphocyte ratio as a novel prognostic factor in patients with triple negative breast cancer. Cancer Manag Res. 2019 May 31;11:5125–5131.

14. Artaç M, Uysal M, Karaağaç M, et al. Prognostic Impact of Neutrophil/Lymphocyte Ratio, Platelet Count, CRP, and Albumin Levels in Metastatic Colorectal Cancer Patients Treated with FOLFIRI-Bevacizumab. J Gastrointest Cancer. 2017 Jun;48(2):176–80.

15. Zhu J, Yamane H, Paul WE, et al. Differentiation of effector CD4 T cell populations (+). Annu Rev Immunol. 2010;28:445–89.
16. Liang Y, He M, Fan X, et al. An abnormal elevation of serum CA72-4 by ganoderma lucidum spore powder. Ann Clin Lab Sci. 2013 Summer;43(3):337–40.
17. Li yilan. qiao shanshan, li guoxing. Effect of ganoderma lucidum powder on anti-tumor and improving immunity [J]. Chinese journal of chronic disease prevention control. 2004;12(4):156–60.[in chinese].
18. Wang yun. Yang jin-ming, wang Yang. Immunomodulatory effects of ganoderma lucidum spores [J]. Chinese edible fungi. 2002;21(1):156–60.[in chinese].
19. Boh B. Boh B. Ganoderma lucidum: a potential for biotechnological production of anti-cancer and immunomodulatory drugs. Recent Pat Anticancer Drug Discov. 2013 Sep;8(3):255–87.
20. Egen JG, Ouyang W, Wu LC. Human Anti-tumor Immunity: Insights from Immunotherapy Clinical Trials. Immunity. 2020 Jan 14;52(1):36–54.
21. Shaw DM, Merien F, Braakhuis A, et al. T-cells and their cytokine production: The anti-inflammatory and immunosuppressive effects of strenuous exercise. Cytokine. 2018 Apr;104:136–42.
22. Bao XF, Zhen Y, Ruan L, et al. Purification, characterization, and modification of T lymphocyte-stimulating polysaccharide from spores of Ganoderma lucidum. Chem Pharm Bull (Tokyo). 2002 May;50(5):623–9.
23. Zhao H, Zhang Q, Zhao L, et al. Spore Powder of Ganoderma lucidum Improves Cancer-Related Fatigue in Breast Cancer Patients Undergoing Endocrine Therapy: A Pilot Clinical Trial. Evid Based Complement Alternat Med. 2012;2012:809614.
24. Speiser DE, Ho PC, Verdeil G. Regulatory circuits of T cell function in cancer. Nat Rev Immunol. 2016 Oct;16(10):599–611.
25. Meloni F, Morosini M, Solari N, et al. Foxp3 expressing CD4 + CD25 + and CD8 + CD28- T regulatory cells in the peripheral blood of patients with lung cancer and pleural mesothelioma. Hum Immunol. 2006;67(1–2):1–12.
26. Karagoz B, Bilgi O, Gumus M, et al. CD8 + CD28- cells and CD4 + CD25 + regulatory T cells in the peripheral blood of advanced stage lung cancer patients. Med Oncol. 2010;27(1):29–33.
27. Li L, Ma Y, Xu Y. Li L, et al. Follicular regulatory T cells infiltrated the ovarian carcinoma and resulted in CD8 T cell dysfunction dependent on IL-10 pathway. Int Immunopharmacol. 2019 Mar;68:81–87. doi: 10.1016/j.intimp.2018.12.051.
28. Li CW, Osman R, Menconi F, et al. Flexible peptide recognition by HLA-DR triggers specific autoimmune T-cell responses in autoimmune thyroiditis and diabetes. J Autoimmun. 2017 Jan;76:1–9.
29. Saraiva DP, Jacinto A, Borralho P, et al. HLA-DR in Cytotoxic T Lymphocytes Predicts Breast Cancer Patients’ Response to Neoadjuvant Chemotherapy. Front Immunol. 2018 Nov 13;9:2605.
30. Tian T, Gu X, Zhang B, et al. Increased circulating CD14(+)HLA-DR−/low myeloid-derived suppressor cells are associated with poor prognosis in patients with small-cell lung cancer. Cancer Biomark. 2015;15(4):425–32.
31. Bilici M, Okcu N, Cayir K, et al. Distribution of HLA Tissue Groups in Patients with Gastric Cancer. Eurasian J Med. 2010 Apr;42(1):9–11.
32. Soda H, Ogawara D, Fukuda Y, et al. Dynamics of blood neutrophil-related indices during nivolumab treatment may be associated with response to salvage chemotherapy for non-small cell lung cancer: A hypothesis-generating study. Thorac Cancer. 2019 Feb;10(2):341–6.
33. Sun LX, Li WD, Lin ZB, Duan XS, Xing EH, Jiang MM, et al. Cytokine production suppression by culture supernatant of B16F10 cells and amelioration by Ganoderma lucidum polysaccharides in activated lymphocytes. Cell Tissue Res. 2015;360(2):379–89. doi:10.1007/s00441-014-2083-6.
34. Che D, Zhang S, Jing Z, et al. Macrophages induce EMT to promote invasion of lung cancer cells through the IL-6-mediated COX-2/PGE(2)/β-catenin signalling pathway. Mol Immunol. 2017 Oct;90:197–210.
35. Mateu-Jimenez M, Curull V, Pijuan L, Sánchez-Font A, et al. Systemic and Tumor Th1 and Th2 Inflammatory Profile and Macrophages in Lung Cancer: Influence of Underlying Chronic Respiratory Disease. J Thorac Oncol. 2017 Feb;12(2):235–48.

36. Guenova E, Watanabe R, Teague JE, et al. TH2 cytokines from malignant cells suppress TH1 responses and enforce a global TH2 bias in leukemic cutaneous T-cell lymphoma. Clin Cancer Res. 2013 Jul 15;19(14):3755–63.

37. Guo X, Zhang Y, Zheng L, et al. Global characterization of T cells in non-small-cell lung cancer by single-cell sequencing. Nat Med. 2018 Jul;24(7):978–85.

38. Bense RD, Sotiriou C, Piccart-Gebhart MJ, et al. Relevance of Tumor-Infiltrating Immune Cell Composition and Functionality for Disease Outcome in Breast Cancer. J Natl Cancer Inst. 2016 Oct 13;109(1):djw192.

39. Nicolet BP, Guislain A, Wolkers MC. Combined Single-Cell Measurement of Cytokine mRNA and Protein Identifies T Cells with Persistent Effector Function. J Immunol. 2017 Jan;15(2):962–70. 198(.

40. Jérôme Galon, Daniela Bruni. Tumor Immunology and Tumor Evolution: Intertwined Histories. Immunity. 2020 Jan 14;52(1):55–81.

41. Wang L, Liang D, Xu X, et al. The prognostic value of neutrophil to lymphocyte and platelet to lymphocyte ratios for patients with lung cancer. Oncol Lett. 2017;14(6):6449–56.

Figures
Figure 1

Flow cytometry analysis of typical NKT, Th and CTL cell subsets in PBMC of patients with breast cancer and lung cancer whether have taken ganoderma spore powder (A is treated group, B is untreated group)
Figure 2

Comparison of the frequencies of T cell subsets among the treated patients group and controls after 6 weeks. Data are presented as median. Numbers on the vertical axis indicate % within T cells. \( *p<0.05 \) vs controls with ANOVA and Bonferroni’s correction.
Figure 3

Univariate COX analysis of AGR, NLR and PFS. A: PFS and AGR of patients without ganoderma spore powder; B: PFS and AGR of patients with ganoderma spore powder; C: PFS and NLR of patients without ganoderma spore powder; D: PFS and NLR of patients with ganoderma spore powder.

Supplementary Files

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- SupplementaryTable1.docx