The spatial distribution of immune cell subpopulations in hepatocellular carcinoma

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Abstract
Infiltrating immune cells in the tumor microenvironment (TME) influence tumor progression and patient prognosis, making them attractive therapeutic targets for immunotherapy research. A deeper understanding of immune cell distributions in the TME in hepatocellular carcinoma (HCC) is needed to identify interactions among different immune cell types that might impact the effectiveness of potential immunotherapies. We performed multiplex immunohistochemistry using a tissue microarray of samples from 302 patients with HCC to elucidate the spatial distributions of immune cell subpopulations (CD3+, CD4+, CD8+, CD66b+, and CD68+) in HCC and normal liver tissues. We analyzed the associations between different immune subpopulations using Pearson's correlation. G(r) functions, K(r) functions and Euclidean distance were applied to characterize the bivariate distribution patterns among the immune cell types. Cox regression and Kaplan-Meier analysis were used to evaluate the associations between tumor infiltration by different immune cells and patient outcomes after curative surgery. We also analyzed the relationship between the spatial distribution of different immune cell subpopulations with HCC patient prognosis. We found that the immune cell spatial distribution in the HCC TME is heterogeneous. Our study provides a theoretical basis for HCC immunotherapy.

KEYWORDS
hepatocellular carcinoma, immune cell subpopulations, multiplex immunohistochemistry, prognosis, tumor microenvironment

Abbreviations: HCC, Hepatocellular carcinoma; IHC, Immunohistochemistry; OS, Overall survival; TME, Tumor microenvironment.

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1 INTRODUCTION

Hepatocellular carcinoma (HCC) has high recurrence and mortality rates, making it one of the most lethal forms of cancer. In early 1990s, cytokine immunotherapy was proposed as a new treatment modality for HCC. Immunotherapy with anti-programmed cell death protein 1 (PD-1) agents has achieved encouraging results against various tumor types, including breast cancer and non-small-cell lung cancer. However, the response rate to immunotherapy in patients with HCC remains unsatisfactory, primarily due to a lack of understanding of the spatial context of the highly heterogeneous tumor microenvironment (TME).

The TME is a cellular environment comprising stromal cells, the extracellular matrix, immune cells, and malignant cells. Tumors depend on the TME for growth because interactions among cellular compartments are vital for both tumor cells and stromal cells. CD8+ T cells, CD4+ T helper 1 (Th1) cells, and the secreted cytokine interferon gamma suppress tumor development, whereas tumor-associated neutrophils recruit macrophages and regulatory T cells to promote tumor growth and resistance to the anti-cancer drug sorafenib. In addition, the PD-1/programmed cell death-ligand 1 (PD-L1) axis interacts with certain mutations in tumor cells to modulate antitumor immunity because upregulation of PD-L1 inhibits T cell effector functions. Importantly, few studies have investigated the spatial relationships among different types of immune cells within the TME.

Multiplex immunohistochemistry (IHC) provides more information than traditional IHC and has emerged as a crucial tool in TME studies. We used multispectral quantitative fluorescent IHC to explore the spatial distributions of immune cells in the TME and found that the distribution of immune cells was heterogeneous between HCC tumor tissues and normal tissues. We also found that the immune cell distribution was associated with patient prognosis.

2 MATERIALS AND METHODS

2.1 Patient information and specimens

We performed multiplex IHC using a tissue microarray of matched tumor and non-tumor liver samples from the 302 patients with HCC who had undergone curative resection at the Liver Cancer Institute, Zhongshan hospital, Fudan University between January 2003 and March 2004. Patients with distant metastasis or any history of treatment for HCC were excluded from the study. All patients were diagnosed with HCC based on postoperative pathology evaluation. The Zhongshan Hospital Research Ethics Committee granted ethical approval for the study of human subjects. The American Joint Committee on Cancer staging system was used to determine the tumor grade in each patient.

2.2 Tissue microarrays and multiplexed immunohistochemistry

We constructed tissue microarrays as previously described using 2 tumor and 2 non-tumor core specimens from each patient, each with a diameter of 1.5 mm. For multiplex IHC analysis of the microarrays, we used the Opal 7-Color Fluorescent IHC Kit in accordance with the manufacturer’s protocol (NELB11001KT; PerkinElmer) with the following fluorescent markers: DAPI (1:100; ab104139; abcam), anti-CD3 (1:100; ab16669; abcam), anti-CD4 (1:500; ab133616; abcam), anti-CD8 (1:200; #85336; CST), anti-CD66b (1:100; ab197678; abcam), and anti-CD68 (1:100; ab783; abcam). Opal multiplexing is a serial IHC method that relies on tyramide single amplification. Opal slides can be visualized using standard fluorescence microscopy. Multispectral imaging allows quantitative unmixing of various fluorophores and removal of tissue autofluorescence. We analyzed fluorescence microscopy images using Inform image analysis software (version 2.4; PerkinElmer). All representative images were chosen by 3 pathologists.

2.3 Quantification of the spatial distribution of immune cells

We used a bivariate point pattern characterized by the bivariate G(r) and K(r) functions to represent the relative spatial distributions of immune cell subpopulations. The bivariate G(r) function is defined as a probability function of the nearest neighbor distance within a given radius and the formula is:

\[ G(r) = \sum_{i,j} \mathbb{1}(d_{ij} \leq r) e_{i,j}/n \]

The bivariate K(r) function is defined as the expected number of cells appearing within the radius and the formula is:

\[ K(r) = (\alpha/(n^2(n-1))) \sum_{i,j \neq \ell} |d_{ij}| \]

In both functions, the distance between 2 points is defined as \(d_{ij}\), the logical decision function within the radius is defined as \(|d_{ij}| \leq r\), \(n\) is the acreage, \(n\) is the count of cells, and \(r\) is the radius of the area in which the function is evaluated. As a null hypothesis, we assume that the different types of immune cells are independent of each other, and that the distances between nearest neighbors from different cell types follow a Poisson distribution.

The Euclidean distance between 2 points \(u = (u_1, u_2)\) and \(v = (v_1, v_2)\) is defined as follows:

\[ ||u - v|| = \left( (u_1 - v_1)^2 + (u_2 - v_2)^2 \right)^{1/2} \]
We calculated the G(r), K(r) functions, and the distance between 2 different immune cell subpopulations using the toolbox ‘spatstat’ in R.

2.4 Statistical analysis

We used SPSS, version 23.0 (IBM Corporation) for all statistical analyses. We performed two-way comparisons between groups using the Student’s t-test and analyzed differences in OS using the Kaplan-Meier method and log-rank test. We used univariate and multivariate Cox proportional hazard models to calculate associations between the survival outcomes and clinicopathologic characteristics. We applied Pearson’s correlation coefficient (R) to evaluate potential correlations between the groups. We used *P < .05 as the threshold for significance in all statistical tests.

3 RESULTS

3.1 Patient and disease characteristics

Our study sample included 302 patients with primary HCC who had undergone curative resection. Most of the patients were male (n = 257, 85.1%) and had a history of hepatitis B virus (HBV) infection (n = 264, 87.4%). The median age was 50 y, and the median tumor diameter was 5.0 cm. In accordance with the American Joint Committee on Cancer staging system, 51.0% (n = 154) of the patients had T1 disease. Post-surgical histologic evaluation of the tumor tissues by 3 independent pathologists determined that most tumors were moderately differentiated (n = 239, 79.1%), and almost half (n = 138, 45.7%) had vascular invasion (Table 1).

3.2 Heterogeneous immune cell subpopulations infiltrated the HCC tumors

We characterized immune cell distribution in the HCC tumors using multiplex IHC, which allowed simultaneous visualization of 5 markers in each formalin-fixed and paraffin-embedded tissue section. To explore the subpopulations of immune cells in HCC, we used fluorescent antibodies to visualize CD3, CD4, CD8, CD66b, and CD68. We also used DAPI as a nuclear stain. In addition to the shape of nucleus, the marker CD3 was used to identify all T cells. We defined CD3+CD8+CD4- cells as cytotoxic T cells, CD3+CD8-CD4+ cells as CD4+ T cells, and any other cells with CD3+ staining that were negative for the other 2 markers as “other T cells” (CD3+CD8-CD4-). We defined CD68+ cells as macrophages and CD66b+ cells as neutrophils. To minimize false-positive results, we applied co-localization in our process to identify the different immune cell populations. Representative images of the different cells in tumor tissue and non-tumor tissues are shown in Figure 1 and Figure S1, respectively.

3.3 Different immune cell subpopulations aggregated together in HCC tumors

When we compared the immune cell distributions between tumor and non-tumor tissues, lower proportions of CD3+ T cells were observed in the tumor tissues (6.45% vs. 2.69%; **P < .001; Figure 2A). Furthermore, the proportion of CD3+ T cells that were CD4- was lower in the non-tumor tissues than in matched tumor tissues (9.53% vs. 16.12%; *P < .05; Figure 2B). Conversely, the proportion of CD3+ T cells that were CD8+ was higher in tumor tissues than in non-tumor tissues (34.58% vs. 30.55%; **P < .001; Figure 2C).

TABLE 1 Patient clinical information (n = 302)

| Clinicopathologic index | ≤50 | >50 |
|-------------------------|-----|-----|
| Age (y)                 | 157 (52.0%) | 145 (48.0%) |
| Sex                     | Male 257 (85.1%) | Female 45 (14.9%) |
| HBsAg                   | Negative 38 (12.6%) | Positive 264 (87.4%) |
| HCV                     | Negative 295 (97.7%) | Positive 7 (2.3%) |
| AFP (ng/ml)             | ≤20 90 (29.7%) | >20 212 (70.3%) |
| GGT (U/L)               | ≤54 124 (41.1%) | >54 178 (58.9%) |
| Liver cirrhosis         | No 33 (10.9%) | Yes 269 (89.1%) |
| Tumor size (cm)         | ≤5 143 (47.4%) | >5 159 (52.6%) |
| Tumor number            | Single 255 (84.4%) | Multiple 47 (15.6%) |
| Microvascular invasion  | Absence 164 (54.3%) | Present 138 (45.7%) |
| Tumor encapsulation     | Complete 149 (49.3%) | None 153 (50.7%) |
| Tumor differentiation   | I-II 239 (79.1%) | III-IV 63 (20.9%) |
| TNM stage               | I 154 (51.0%) | II-II 148 (49.0%) |

Note: AFP, alpha-fetoprotein; GGT, gamma glutamyl transferase; TNM, tumor-node-metastasis.
(R = 0.440; **P < .001; Figure 3A). Strikingly, the presence of neutrophils in tumor tissues was also correlated with that of CD4⁺ T cells (R = 0.183; *P < .001; Figure 3A).

Carstens and colleagues used K-functions and L-functions (the deformed variants of K-functions) to characterize the spatial patterns of pancreatic cancer cells and intratumoral T cells.¹⁴ We combined G(r) and K(r) functions to make our results more reliable (Figure 3B-E). The blue line represents the theoretical distribution of distances between cells in 2 different immune subpopulations, which we assume conforms to a Poisson distribution. The red line represents the actual distribution of those distances. Values of r ranging from 0 to 30 μm are considered ideal to calculate the spatial relationship between 2 cell populations.¹⁵ When the red line is above the blue line, the aggregation patterns of 2 the cell types under consideration cannot be considered independent. Conversely, if the red line was below the blue line, the aggregation patterns of the 2 cell types can be considered independent. In the plot of the G(r) and K(r) functions for macrophages and neutrophils, the red line is above the blue line, indicating that macrophages and neutrophils tended to aggregate closer together than would be expected if their spatial distributions were independent (Figure 3C). The aggregation patterns of CD8⁺ T cells with neutrophils and CD4⁺ T cells with neutrophils were not independent of each other in HCC (Figure 3D,E). The spatial distributions of different immune cell populations in non-tumor tissues are shown in Figure S2.

### 3.4 Immune cell infiltration correlated with HCC prognosis

We used patient follow-up information to further explore the correlation between infiltrating immune cells and patient outcomes.
FIGURE 3 Associations between different immune cell types. A, Intratumoral spatial distribution of CD66b+ cells (neutrophils) was positively correlated with those of CD8+ T cells ($R = 0.145$, *$P < .05$), CD68+ cells (macrophages) ($R = 0.440$, **$P < .001$), and CD4+ T cells ($R = 0.183$, **$P < .001$). B, Cell phenotype map showing the spatial distributions of different immune cell populations. C-E, $G(r)$ and $K(r)$ functions were used to characterize the observed and expected aggregation patterns between (C) CD66b+ cells and CD68+ cells, (D) CD66b+ cells and CD8+ cells, and (E) CD66b+ cells and CD4+ cells.
after surgical resection of HCC (Figure 4). Higher infiltrations of CD3⁺ T cells and CD8⁺ T cells were associated with better OS and lower cumulative recurrence rates (**P < .001 and *P < .05, **P < .001 for both, respectively; Figure 4A). Conversely, the presence of neutrophils and macrophages within HCC tumors was negatively correlated with OS and the cumulative recurrence rates (**P < .001 for both, *P < .001 and *P < .05, respectively; Figure 4A). However, the presence of CD4⁺ T cells within tumor was not associated with OS and cumulative recurrence rates (P > .05 for both, Figure 4A). The association of different immune cells in non-tumor liver tissues with patient prognosis is shown in Figure S3.

We calculated the average distance between 2 different immune cell subpopulations, and values of r ranging from 0 to 30 μm. The mean distances between neutrophils and macrophages, neutrophils and CD4⁺ T cells, and neutrophils and CD8⁺ T cells were 18.08, 20.28, and 19.59 μm, respectively. Furthermore, a shorter distance (S-D) between neutrophils and macrophages was associated with worse OS and higher cumulative recurrence rates (**P < .001 for both; Figure 4B). However, the distance between neutrophils and CD4⁺ T cells and neutrophils and CD8⁺ T cells was not correlated with OS or cumulative recurrence rates (P > .05 for both; Figure 4B).

Univariate and multivariate Cox proportional hazard models revealed that tumor size, microvascular invasion, and the presence of CD8⁺, CD66b⁺ and CD68⁺ cells in tumor tissues were independent prognostic factors for both OS and the time to recurrence (Table 2).
The most common underlying cause of HCC is chronic infection with HBV or hepatitis C virus.\textsuperscript{16} The efficacy of immunology therapy in the treatment of patients with HCC remains unsatisfactory. One reason is that our understanding of the complex mechanisms by which HCC cells escape the body's immune response remains incomplete. Cancer progression is regulated by interactions between cancer cells and the surrounding microenvironment,\textsuperscript{17} and the immune system plays dual roles in malignancy development and progression.\textsuperscript{18} Some immune cells inhibit tumor growth, whereas others enhance malignancy progression.\textsuperscript{19} To characterize the spatial distribution of immune cells, we performed multiplex IHC using tissue microarrays from 302 patients with primary HCC. Our results showed that the spatial distribution of immune cells is heterogeneous in the HCC TME.

### TABLE 2 Univariate and multivariate analyses of prognostic factors in HCC (n = 302)

| Variable                                      | TTR                  | HR (95% CI)       | P       | OS                     | HR (95% CI)       | P       |
|-----------------------------------------------|----------------------|-------------------|---------|------------------------|-------------------|---------|
| **Univariate analysis**                       |                      |                   |         |                        |                   |         |
| Age, y (≤50 vs >50)                           | 0.918                | (0.675-1.248)     | .584    | 0.754 (0.547-1.040)     | .086              |         |
| Sex (female vs male)                          | 1.767                | (1.082-2.885)     | .023    | 1.640 (0.977-2.755)     | .061              |         |
| HBsAg (negative vs positive)                  | 1.118                | (0.700-1.786)     | .640    | 1.064 (0.683-1.658)     | .783              |         |
| AFP, ng/ml (≤20 vs >20)                       | 1.128                | (0.807-1.576)     | .482    | 1.587 (1.091-2.309)     | .016              |         |
| GGT, U/L (≤54 vs >54)                         | 1.304                | (0.950-1.789)     | .101    | 1.647 (1.174-2.313)     | .004              |         |
| Liver cirrhosis (no vs yes)                   | 1.230                | (0.723-2.093)     | .444    | 1.305 (0.739-2.305)     | .359              |         |
| Tumor size, cm (≤5 vs >5)                     | 1.650                | (1.208-2.254)     | .002    | 2.139 (1.530-2.990)     | .000              |         |
| Tumor number (single vs multiple)             | 1.388                | (0.922-2.089)     | .116    | 1.605 (1.080-2.384)     | .019              |         |
| Microvascular invasion (no vs yes)            | 1.962                | (1.438-2.676)     | .000    | 2.488 (1.795-3.449)     | .000              |         |
| Tumor encapsulation (complete vs none)        | 1.718                | (1.260-2.343)     | .001    | 1.747 (1.262-2.417)     | .001              |         |
| Tumor differentiation (I + II vs III +IV)      | 1.198                | (0.830-1.729)     | .335    | 1.490 (1.034-2.145)     | .032              |         |
| TNM stage (I vs II III)                       | 1.195                | (0.879-1.624)     | .256    | 1.482 (1.075-2.042)     | .016              |         |
| CD3 (low vs high)                             | 0.713                | (0.519-0.981)     | .038    | 0.672 (0.489-0.925)     | .015              |         |
| CD4 (low vs high)                             | 0.782                | (0.569-1.073)     | .128    | 0.800 (0.583-1.098)     | .168              |         |
| CD8 (low vs high)                             | 0.370                | (0.265-0.518)     | .000    | 0.348 (0.249-0.488)     | .000              |         |
| CD66b (low vs high)                           | 2.784                | (1.981-3.914)     | .012    | 3.137 (2.230-4.413)     | .000              |         |
| CD68 (low vs high)                            | 1.764                | (1.276-2.440)     | .001    | 1.798 (1.301-2.484)     | .000              |         |
| **Multivariate analysis**                     |                      |                   |         |                        |                   |         |
| Sex (female vs male)                          | 2.171                | (1.255-3.757)     | .006    | NA                     | NA                | NA      |
| AFP, ng/ml (≤20 vs >20)                       | NA                   | NA                |         | 1.328 (0.895-1.969)     | .158              |         |
| GGT, U/L (≤54 vs >54)                         | NA                   | NA                |         | 1.458 (1.024-2.077)     | .037              |         |
| Tumor size, cm (≤5 vs >5)                     | 1.783                | (1.247-2.549)     | .002    | 1.820 (1.280-2.587)     | .001              |         |
| Tumor number (single vs multiple)             | NA                   | NA                |         | 1.033 (0.675-1.579)     | .883              |         |
| Microvascular invasion (no vs yes)            | 1.480                | (1.048-2.090)     | .026    | 1.680 (1.187-2.379)     | .003              |         |
| Tumor encapsulation (complete vs none)        | 1.302                | (0.982-1.828)     | .127    | 1.111 (0.786-1.570)     | .550              |         |
| Tumor differentiation (I + II vs III +IV)      | NA                   | NA                |         | 1.228 (0.882-1.711)     | .224              |         |
| TNM stage (I vs II III)                       | NA                   | NA                |         | 1.143 (0.867-1.506)     | .345              |         |
| CD3 (low vs high)                             | 1.706                | (0.493-1.009)     | .056    | 0.673 (0.471-0.963)     | .030              |         |
| CD8 (low vs high)                             | 0.297                | (0.208-0.425)     | .000    | 0.272 (0.189-0.390)     | .000              |         |
| CD66b (low vs high)                           | 3.138                | (2.182-4.514)     | .000    | 3.326 (2.306-4.798)     | .000              |         |
| CD68 (low vs high)                            | 2.039                | (1.422-2.923)     | .000    | 2.391 (1.665-3.435)     | .000              |         |

Note: Cox proportional hazards regression model. AFP, alpha-fetoprotein; CI, confidential interval; GGT, gamma glutamyl transferase; HR, hazard ratio; NA, not adopted.

4 | DISCUSSION

The most common underlying cause of HCC is chronic infection with HBV or hepatitis C virus.\textsuperscript{16} The efficacy of immunology therapy in the treatment of patients with HCC remains unsatisfactory. One reason is that our understanding of the complex mechanisms by which HCC cells escape the body’s immune response remains incomplete. Cancer progression is regulated by interactions between cancer cells and the surrounding microenvironment,\textsuperscript{17} and the immune system plays dual roles in malignancy development and progression.\textsuperscript{18} Some immune cells inhibit tumor growth, whereas others enhance malignancy progression.\textsuperscript{19} To characterize the spatial distribution of immune cells, we performed multiplex IHC using tissue microarrays from 302 patients with primary HCC. Our results showed that the spatial distribution of immune cells is heterogeneous in the HCC TME.

The HCC tumor tissues contained fewer CD3\textsuperscript{+} T cells than the non-tumor tissues, a finding consistent with previous studies.\textsuperscript{20,21} Among T lymphocytes, CD8\textsuperscript{+} T lymphocytes were the predominant T-cell population. CXCR3-mediated CD8\textsuperscript{+} T cell trafficking into tumor tissues was associated with favorable outcomes in patients with cancers such as melanoma and ovarian cancer.\textsuperscript{22} In the present study, HCC patients with higher rates of CD8 expression...
on tumor-infiltrating T cells had better outcomes after surgery. In addition, the percentage of CD4+ T cells within the CD3+ T-cell population was much higher in HCC tumors than in normal liver tissues. Importantly, CD4+ T cells play a critical role in antitumor immune function. Conventional effector CD4+ T cells enhance the ability of the immune system to eliminate tumor cells by stimulating pro-inflammatory programs and licensing dendritic cells.23

Neutrophils are the first responders in the host immune defense against infection.24 In the progression of primary tumors, chronic inflammation is a common aspect. The role of neutrophils in tumor biology has remained controversial, however.25 Previous studies have shown that tumor patients with low numbers of tumor-infiltrating neutrophils have a better OS than those with a high number of tumor-infiltrating neutrophils.26, 27 Consistent with those results, we discovered that increased numbers of neutrophils were associated with poor outcomes in HCC patients.

Macrophages are a large component of the infiltrating leukocyte compartment in the TME. Tumor-associated macrophages also exert immunosuppressive functions.28 Kuang and colleagues reported that macrophages suppressed the response of T cells through PD-L1 in HCC.29 We also discovered that patients with a low number of macrophages had a better prognosis.

In our previously studied case, we found tumor-associated neutrophils recruited macrophages to promote tumor progression by secreting CCL2.30 The results of G(r) and K(r) functions supported the observation that neutrophils and macrophages aggregated closer together. Furthermore, we discovered that a longer distance between neutrophils and macrophages was associated with a better prognosis. In TME, CD8+ T cells and neutrophils play different roles in predicting patient prognosis. Some groups have reported that neutrophils play a critical role in recruiting of CD8+ T cells and frequently colocalize with CD8+ T cells in colorectal cancer.31–33 In our study, G(r) and K(r) functions indicated that CD8+ T cells and neutrophils tended to aggregate closer together. However, the distance between neutrophils and CD8+ T cells was not correlated with patient prognosis after surgery. We suggest that a mechanism of interaction exists between a certain subgroup of CD8+ T cells and neutrophils, but this does not influence neutrophils and CD8+ T cells as independent prognostic predictors. The cross-talk between neutrophils and CD8+ T cells has not been investigated. Therefore, our future studies could explore the mechanism of interaction between CD8+ T cells and neutrophils.

Multiplex IHC allows the evaluation of different cell subsets on a single slide. Two tumor cores and 2 cores of normal liver tissues were available for each patient. In addition, false-positive results are a common problem in single-marker IHC studies. Our study used co-localization to improve the accuracy of the experimental results. The use of multiple markers allows the elucidation of different immune cell subsets in a single tissue section. This study is the first to use G(r) and K(r) functions to simultaneously characterize the spatial relationships among different immune cell subpopulations in HCC tumors and analyze the association of the distance between 2 different immune cells with patients’ prognosis using Euclidean distance.

Our study illustrated the spatial distribution of several critical immune cell types. In the past decades, all researchers have reached a consensus that tumor cells grow in a complex tissue microenvironment. Sia and colleagues presented gene profiles of HCC TME using a non-negative matrix factorization algorithm and discovered a novel immune specific class which might be an ideal candidate for an immunotherapy target, such as the PD-L1 inhibitor.34 We did not take a deeper exploration to represent a comprehensive characterization of HCC TME. So, we need to validate our research in a larger patient cohort and focus on the mechanism that may provide a theoretical basis for clinical treatment.

In summary, interactions between tumor cells and immune cells and the relationships between different immune cell subpopulations are essential properties of the TME. Our results provide a picture of the TME immune cell composition. The mechanisms of the pathophyslogic interactions in the TME need to be explored further.

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DISCLOSURE
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SUPPORTING INFORMATION
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