METHOD FOR THE PROGNOSIS OF SURVIVAL TIME OF A PATIENT SUFFERING FROM A SOLID CANCER

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Abstract
The present invention relates to an in vitro method for the prognosis of the survival time of a patient suffering from a solid cancer, comprising the quantification of the cell density of follicular B cells present in tumor-induced lymphoid structures from said patient, wherein a high density of follicular B cells indicates that the patient has a favorable prognosis and a low density of follicular B cells indicates that the patient has a poor prognosis.
Figure 1

- LN
- NSCLC

A. IgD (HE)
B. NSCLC

C. CD20/CD23
D. CD20/CD23

E. AID (HE)
F. NSCLC

G. CD20 / Ki67
H. NSCLC
Figure 2C

Bar charts showing the percentage of memory B cells, GC-B cells, and naive B cells in different Bm categories (Bm1, Bm2, Bm3, Bm4, Bm5) with early and late stages.
Event/total:

- DC-LAMP low: 20/27 patients
- DC-LAMP high: 12/29 patients

$p = 0.0373$

**Figure 4**
Figure 5

A. 

Follicular B cells

- Foll-CD20 High (n=49)
- Foll-CD20 low (n=73)

OS probability (%)

P = 0.007

Time (months)

B. 

Mature DC

- DC-Lamp High (n=52)
- DC-Lamp low (n=70)

OS probability (%)

P = 0.04

Time (months)

C. 

Follicular B cells and mature DC

- Foll-CD20/DC-Lamp High (n=27)
- Foll-CD20/DC-Lamp mix (n=47)
- Foll-CD20/DC-Lamp low (n=48)

OS probability (%)

P = 0.003

Time (months)
METHOD FOR THE PROGNOSIS OF SURVIVAL TIME OF A PATIENT SUFFERING FROM A SOLID CANCER

FIELD OF THE INVENTION

[0001] The present invention relates to an in vitro method for the prognosis of survival time of a patient suffering from a solid cancer.

BACKGROUND OF THE INVENTION

[0002] As indicated in Dieu-Nosjean et al. (J Clin Oncol 26:4410-4417, 2008), lung cancer is the most common cause of cancer related death in the world. Approximately 80% to 90% of cases involve Non-Small-Cell Lung Cancer (NSCLC), which includes adenocarcinoma and squamous cell carcinoma. Only patients whose tumors can be completely resected have a significant chance of increased survival. However, as many as 30% of patients with stage I disease experience recurrence after surgery. The correlation between tumor-infiltrating immune cells and the prognosis of patients with lung cancer is controversial.

[0003] A tumor is composed of malignant, stromal, endothelial, and immune cells that form a heterogeneous network and exhibit complex interactions. Although tumor eradication by the immune system is often inefficient, there is evidence that many developing cancers are not ignored by the immune system. Spontaneous tumor regressions occurring concomitantly with autoimmune manifestations and the higher incidence of tumors in immunosuppressed patients are indications of the involvement of the immune system in tumor rejection. Mice deficient in immune functions spontaneously develop tumors. The density of tumor-infiltrating lymphocytes (TILs) with cytotoxic and memory phenotypes is highly predictive of good clinical outcome. However, although prognosis is related to the homing of effector immune cells, it is still unclear where the activation of the specific immune response takes place: in the tumor, the draining lymph node, or both.

[0004] It is now well established that immune responses can take place at distance of secondary lymphoid organs, in tertiary lymphoid structures (TLS). Dieu-Nosjean et al. have observed that these lymph node-like structures can develop in lung cancer patients. They have been named “Tumor-induced Bronchus-Associated Lymphoid Tissues” (Ti-BALT) as they were never found in the non-tumoral tissues of NSCLC patients. Moreover, Dieu-Nosjean et al. have demonstrated that the density of mature DC, a population which was selectively detected in Ti-BALT, is associated with a favorable clinical outcome, suggesting that they represent an activation site for tumor-specific T cells.

[0005] The presence of TLS has been reported in other human tumors (e.g., colorectal and breast (Gibert et al., Cancer Res 2009; 69(5) 2000-2009)) indicating that ectopic lymphoid structures arise in many solid tumors.

SUMMARY OF THE INVENTION

[0006] The present invention relates to an in vitro method for the prognosis of the survival time of a patient suffering from a solid cancer, comprising the quantification of the cell density of follicular B cells present in tumor-induced lymphoid structures from said patient, wherein a high density of follicular B cells indicates that the patient has a favorable prognosis and a low density of follicular B cells indicates that the patient has a poor prognosis.

[0007] The present invention provides a novel method for the prognosis of the survival time of solid cancer patients. The method is of higher accuracy than currently used staging methods (e.g., UICC-TNM) and thus fulfills a long-felt and ongoing need in the art to correctly and accurately predict the likely course or outcome of cancer in a patient, as reflected in survival time. The ability to do so enables medical practitioners to individually adapt cancer treatment protocols to particular patients. Patients who, according to the present method, have a high probability of a good therapy outcome may not need to receive the most aggressive treatments in order to experience a favorable outcome, and thus can avoid or minimize the side effects associated with such treatments, whereas patients with a poor prognosis can be treated aggressively at the earliest possible stage of the disease or by another therapy than the one used.

DETAILED DESCRIPTION OF THE INVENTION

[0008] The present invention relates to an in vitro method for the prognosis of survival time of a patient suffering from a solid cancer, comprising the following steps:

[0009] a) quantifying on the whole tumor, the cell density of follicular B cells, and

[0010] b) comparing the cell density of follicular B cells value obtained at step a) with a predetermined reference value; and

[0011] c) providing a favorable prognosis of survival time for said patient when the cell density of follicular B cells is higher than said predetermined reference value, or providing a poor prognosis of survival time for said patient when the cell density of follicular B cells is lower than said predetermined reference value.

[0012] By tumor-induced lymphoid structure, it is meant the organization of tumor-infiltrating leukocytes into lymph-node-like structure in the stroma of the tumor mass and, is composed of mature dendritic cell-T cell clusters (T-cell areas) and B-cell follicles (B-cell areas). Typically, depending on the tumor section, only one out of the two areas or both areas can be observed.

[0013] By follicular B cells, it is meant B cell subsets clustered into B-cell follicle. They are mainly of naïve and germinal center phenotype.

[0014] The survival can be the disease-specific survival (DSS), the disease-free survival (DFS) or the overall survival (OS).

[0015] The present invention enables the evaluation of the risk of recurrence of a patient who has been surgically treated and, if needed, subsequently received the appropriate treatment (such as radiotherapy, chemotherapy and/or hormonal therapy).

[0016] The method of prognosis according to the invention may be used alone or in combination with any other methods already used for the prognostic assessment of solid cancers, including stage, demographic and anthropometric parameters, results of routine clinical or laboratory examination, including size of the tumor, histopathologic grading, hormone receptors, oncotype . . .

[0017] In a preferred embodiment, the method of the invention further comprises the steps of:

[0018] a) quantifying on the whole tumor, the cell density of mature dendritic cells, and
comparing the cell density of mature dendritic cells value obtained at step a) with a predetermined reference value; and

c) providing a favorable prognosis of survival time for said patient when the cell densities of both the follicular B cells and of the mature dendritic cells are higher than said predetermined reference values,

providing a poor prognosis of survival time for said patient when the cell densities of both follicular B cells and of the mature dendritic cells are lower than said predetermined reference values, or

providing an intermediate prognosis of survival time for said patient when one value of the cell densities of follicular B cells and of the mature dendritic cells is lower than said predetermined reference value and the other value is higher than said other predetermined reference value.

By mature dendritic cells, it is meant a population of dendritic cells that are professional for the presentation of processed antigens to T cells. Mature dendritic cells infiltrating the tumor are selectively located in contact with T cells, in the T-cell rich areas of the tumor-induced lymphoid structure.

Typically follicular B cells density and the mature dendritic cells density may be measured for example by immunohistochemistry performed on a tumor sections (frozen or paraffin-embedded tissue sections) of sample obtained by biopsy.

In an embodiment of the invention, total B cells are detected by immunohistochemistry with an antibody against the CD20 molecule. Among total B cells, follicular B cells are selectively counted on the whole tumor section.

In an embodiment of the invention, mature dendritic cells are detected by immunohistochemistry with an antibody against the DC-Lamp (CD208) molecule. Mature dendritic cells are counted on the whole tumor section. The density of cells may be expressed as the number of cells that are counted per one unit of surface area of the tumor section, e.g. as the number of cells that are counted per intermediate-power field (original magnification x100) or mm² of surface area of the tumor.

As the follicular B cells are organized into a cell aggregate in the B-cell follicle and as they represent more than 98% of total cells present in the B-cell follicle, the density of follicular B cells can also be measured as a total surface of B-cell follicles per one unit of surface area of the tumor, e.g. as the surface area of B-cell follicles in mm² per intermediate-power field (original magnification x100) or mm² of surface area of the tumor.

Typically, the predetermined reference values for the cell density of follicular B cells and for the cell density of mature dendritic cells may be determined by applying statistical methods in large-scale studies on cancer patients.

Examples of solid cancers with tumor-induced lymphoid structures are lung cancers, colorectal cancers and breast cancers.

In a preferred embodiment, the solid cancer is a lung cancer.

In a more preferred embodiment, the solid cancer is a non-small cell lung cancer.

In an embodiment of the invention, the patient is a patient with an early stage of cancer, such as stage I cancer.

In an alternative embodiment of the invention, the patient is a operable patient with advanced stage of cancer (up to stage IIIb cancer).


In an embodiment of the invention, the patient is a patient with early stage of cancer who did not receive any neo-adjuvant, nor adjuvant therapy, such as chemotherapy and/or radiotherapy.

In an alternative embodiment of the invention, the patient is a patient with advanced stage of cancer who receives adjuvant therapy with or without neo-adjuvant therapy, such as chemotherapy and/or radiotherapy.

In the following, the invention will be illustrated by means of the following examples as well as the tables and figures.

FIGURE LEGENDS

FIG. 1. Characterization of B Cell Subsets Into and Outside Ti-BALT

FIG. 2. Comparison of the Subsets of B Cells Infiltrating Lung Tumors, Conventional Secondary Lymphoid Organs and Peripheral Blood

Flow cytometry analysis of CD19+ CD14+ B-cell subsets on lung tumors (n=7), lymph nodes (n=3) and peripheral blood (n=5). Representative dot plots (A) and means (B) of B-cell subsets based on the expression of IgD and CD38 on the 3 localizations. Each bar represents a mean+/− SD of different samples. Statistical significance of B-cell subsets between sites was calculated by Mann-Whitney test. * P<0.05. (C) Comparison of the ratio of the different differentiation stages of naïve B cells (CD23− CD27− CD38− Bm1 and CD23+ CD27− CD38− Bm2, left panel), germinal centre B cells (CD23− CD27− CD77+ CD38+ Bm3 and CD23− CD27− CD77− CD38+ Bm4, centre panel) and memory B cells (CD23− CD27+ CD38+ early Bm5 and CD23− CD27+ CD38− late Bm5, right panel). Abbreviations: pre-GC, pre-germinal centre; GC, germinal centre.

FIG. 3. Prognostic Value of Ti-BALT B Cells in NSCLC Patients

(A) Correlation between the density of DC-Lamp+ mature DC and the density of follicular CD20+ B cells, both populations located in Ti-BALT. Kaplan-Meier curves of disease-specific survival for 74 patients with early-stage NSCLC according to the density of CD20+ follicular B cells (B), the density of DC-Lamp+ mature DC (C), and the density of both cell populations (D). P value was determined using the log-rank test. Abbreviation: DSS, Disease-Specific Survival.

FIG. 4. Prognostic Value of Mature DC in Late-Stage NSCLC Patients Who Received Neo-Adjuvant Chemotherapy.

Kaplan-Meier curves of disease-specific survival of 56 patients according to the density of tumor-infiltrating DC-Lamp+ mature DC. Significant differences between the two groups of patients were evaluated using the log-rank test. The
median DSS was 17 months for the patients with DC-Lamp low tumors, whereas it was 36 months for the patients characterized as having DC-Lamp High tumors. P value significant when <0.05.

**FIG. 5:** Prognostic Value of Tumor-Infiltrating Follicular B Cells and/or Mature DC in Late-Stage NSCLC Patients Who Received Neo-Adjuvant Chemotherapy.

Kaplan-Meier curves of overall survival of 122 patients with advanced-stage of NSCLC and treated by neo-adjuvant chemotherapy according to the presence of a high or low density of tumor-infiltrating (A) CD20+ follicular B cells, (B) DC-Lamp+ mature DC, or (C) both CD20+ follicular B cells and DC-Lamp+ mature DC. Significant differences between the groups of patients were evaluated using the Log-rank test. P value significant when <0.05. Abbreviation: OS, Overall Survival.

**TABLES**

<table>
<thead>
<tr>
<th><strong>Density of follicular CD20+ B cells</strong></th>
<th><strong>Folli-B cells low</strong></th>
<th><strong>Folli-B cells High</strong></th>
<th><strong>Significance</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>mean ± SEM</td>
<td>0.01 ± 0.002</td>
<td>0.097 ± 0.014</td>
<td>&lt;0.0001</td>
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<td>range</td>
<td>0.000-0.029</td>
<td>0.031-0.442</td>
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<tr>
<td>Gender</td>
<td>male:female</td>
<td>34:4</td>
<td>36:2</td>
</tr>
<tr>
<td>Age</td>
<td>67 ± 1</td>
<td>67 ± 2</td>
<td>0.9665</td>
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<td>range</td>
<td>40-83</td>
<td>43-81</td>
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<td>Smoking history</td>
<td>current/never smokers</td>
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<td>pack-year (years) ± SEM</td>
<td>45 ± 4</td>
<td>45 ± 5</td>
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<tr>
<td>range</td>
<td>0-110</td>
<td>0-110</td>
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<tr>
<td>Histological type</td>
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<tr>
<td>ADC</td>
<td>24</td>
<td>63</td>
<td>61</td>
</tr>
<tr>
<td>SCC</td>
<td>14</td>
<td>37</td>
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<tr>
<td>Tumor differentiation</td>
<td>well</td>
<td>19</td>
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<td>intermediate</td>
<td>10</td>
<td>26</td>
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<tr>
<td></td>
<td>poorly</td>
<td>9</td>
<td>24</td>
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<td>pTNM stage</td>
<td>pT1N0(M0)</td>
<td>27</td>
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<td></td>
<td>pT2N(M0)</td>
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<td></td>
<td>pT3N(M0)</td>
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<td>11</td>
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<td>% fibrosis</td>
<td>mean ± SEM</td>
<td>22 ± 3</td>
<td>28 ± 3</td>
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<td></td>
<td>range</td>
<td>0-75</td>
<td>0-85</td>
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<tr>
<td>% necrosis</td>
<td>mean ± SEM</td>
<td>13 ± 3</td>
<td>11 ± 3</td>
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<td>range</td>
<td>1-75</td>
<td>1-50</td>
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<tr>
<td>% Ki67+ tumor cells</td>
<td>mean ± SEM</td>
<td>36 ± 4</td>
<td>36 ± 4</td>
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<td>range</td>
<td>1-80</td>
<td>2-80</td>
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<tr>
<td>Density of DC-Lamp+ mature DC</td>
<td>mean ± SEM</td>
<td>2.652 ± 0.444</td>
<td>7.049 ± 1.034</td>
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<tr>
<td></td>
<td>range</td>
<td>0-12.935</td>
<td>0.500-31.667</td>
</tr>
<tr>
<td>Density of CD3+ T cells (centre of tumor)</td>
<td>mean ± SEM</td>
<td>1.579 ± 0.184</td>
<td>1.931 ± 0.206</td>
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<tr>
<td></td>
<td>range</td>
<td>0.000-5.000</td>
<td>0.000-5.000</td>
</tr>
<tr>
<td>Density of CD3+ T cells (invasive margin)</td>
<td>mean ± SEM</td>
<td>1.487 ± 0.112</td>
<td>2.542 ± 0.172</td>
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<td></td>
<td>range</td>
<td>0.000-3.500</td>
<td>0.500-5.000</td>
</tr>
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</table>

Abbreviations: NSCLC, Non-Small Cell Lung Cancer; ADC, Adenocarcinoma; SCC, Squamous Cell Carcinoma; pTNM, pathologic TNM. All parameters were evaluated among 74 early-stage NSCLC. P-values were obtained using the Fisher’s and the Bonferroni-Dunn exact tests. Abbreviation: SEM, Standard Error of Measurements.
TABLE 2
Clinical and pathological features of NSCLC patients included in the retrospective study

| Pathologic staging and histologic types of lung cancer were determined according to the TNM staging system (Piomonte, NY, Wiley-Liss, 2002) and to the histologic classification of the WHO (Brandoabilla et al., Eur Respir J, 2001), respectively. |

<table>
<thead>
<tr>
<th>Gender</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>60/14</td>
</tr>
</tbody>
</table>

| Age (years) | 64 ± 1 | 41-79 |

| Smoking history | Current/never smokers | 67/7 | 91/9 |
| Pack-years (years) | 45 ± 3 | 0-100 |

| Vital status of patients | Alive | 54/73 |
| Disease-free | 51/65 |
| Dead | 20/27 |
| From metastasis of NSCLC | 9/11 |
| From other causes | 11/12 |

| ADC | 46/62 |
| SCC | 28/38 |

| pTNM stage | pT1NM0 | 48/65 |
| pT2NM0 | 14/19 |
| pT3NM0 | 12/16 |

| Tumor differentiation | Well | 28/38 |
| Intermediate | 22/30 |
| Poorly | 24/32 |

| % Fibrosis | Mean ± SEM | 25 ± 2 | 0-85 |
| Necrosis | Mean ± SEM | 12 ± 2 | 1-75 |
| K67+ tumor cells* | Mean ± SEM | 36 ± 3 | 1-80 |

Abbreviations: NSCLC, Non-Small Cell Lung Cancer; ADC, Adenocarcinoma; SCC, Squamous Cell Carcinoma; pTNM, Pathologic TNM.

TABLE 3-continued
Prognostic parameters for survival in univariate analysis for patients with advanced disease and treated by neo-adjuvant chemotherapy.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological subtype</td>
<td>0.90</td>
<td>0.79 to 1.02</td>
<td>0.109</td>
</tr>
<tr>
<td>Chemotherapy drugs</td>
<td>0.92</td>
<td>0.71 to 1.20</td>
<td>0.548</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>0.90</td>
<td>0.31 to 0.81</td>
<td>0.0044</td>
</tr>
<tr>
<td>Time between chemotherapy and surgery</td>
<td>0.96</td>
<td>0.64 to 1.42</td>
<td>0.426</td>
</tr>
<tr>
<td>% Visible tumor cells</td>
<td>1.66</td>
<td>1.05 to 2.63</td>
<td>0.0313</td>
</tr>
<tr>
<td>TNM stage after chemotherapy</td>
<td>1.53</td>
<td>1.12 to 2.08</td>
<td>0.00678</td>
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<tr>
<td>Immune parameters</td>
<td>CD20+ cell density</td>
<td>0.41</td>
<td>0.24 to 0.70</td>
</tr>
<tr>
<td>Immune score</td>
<td>0.50</td>
<td>0.36 to 0.72</td>
<td>0.000097</td>
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</tbody>
</table>

EXAMPLES

[0049] In the following description, all molecular biology experiments for which no detailed protocol is given are performed according to standard protocol.

[0050] Abbreviations

[0051] ADC, adenocarcinoma; BALT, Bronchus-Associated Lymphoid Tissue; DC, Dendritic Cell; NSCLC, Non-Small-Cell Lung Cancer; SCC, Squamous-Cell Carcinoma; TIL, Tumor-Infiltrating Lymphocyte.

Example 1

[0052] Summary

[0053] The aim of the present study was to determine whether a protective humoral immune response takes place within Ti-BALT.

[0054] Here, we studied B-cell differentiation and migration to Ti-BALT by using complementary approaches (immunohistochemistry, flow cytometry, laser-capture microdissection, PCR low density array) on a series of 104 NSCLC patients. We have shown that B cell follicles of Ti-BALT present the same cellular composition and organization as in canonical secondary lymphoid organs. All stages of differentiation could be detected among tumor-infiltrating B cells. Interestingly, the major B cell compartments were compartments comprising effector cells (memory B cells and plasma cells). We have also shown that the somatic mutation and isotype switching machineries are activated in B cell follicles of Ti-BALT in accordance with the presence of Bm3 and Bm4 cells. We have also studied B cell recruitment to Ti-BALT in order to identify chemotactants orchestrating this migration. Interestingly, intra-tumoral PNA+ high endothelial venules were exclusively associated with Ti-BALT. The PNA+ ligand, CD62L, was selectively detected on most Ti-BALT lymphocytes including all B cells but not on germinal center B cells, as reported for conventional secondary lymphoid organs. The analysis of the chemokine receptor profiles indicated that CXCR5 is expressed by naïve B cells, which parallels the expression of its unique ligand, CXCL13 in the B-cell areas of Ti-BALT. Finally, CXCR5 expression decreased on fully differentiated B cells, a known key regulatory process that allows effector cells to leave the germinal centre, as previously described in lymph nodes.

[0055] Finally, we demonstrated that the density of Ti-BALT B cells is correlated with a long-term survival for lung cancer patients.
All together, these data indicate that Ti-BALT present strong similarity with canonical lymphoid organ, a site specialized in the induction and memory establishment of adaptive immune responses. Thus, Ti-BALT represents an active site for the initiation of a protective humoral immunity. The quantification of follicular B-cells would allow the identification of high-risk patients.

Patients

Fresh and paraffin-embedded lung tumor samples were obtained from NSCLC patients undergoing surgery at Institut Mutualiste Montsouris, Hotel Dieu, Tenon and European Georges Pompidou Hospitals (Paris, France). Pre-operative evaluation of patients included lung, brain, and adrenal CT scan and liver ultrasound echography. They all underwent complete surgical resection of their tumors, including multilevel lymph node sampling or lymphadenectomy, but none received pre-operative chemotherapy or radiotherapy. Patients with an Eastern Cooperative Oncology Group performance status (Finkielstein et al., JCO, 1988) ≤1 were eligible. For the retrospective study, paraffin-embedded tumor biopsies with representative areas of tumor and adjacent lung parenchyma were retrieved from 74 successive patients diagnosed between 1998 and 2002 with early-stage NSCLC (Mountain, Cancer Chest, 1997). The main clinical and pathological features of the patients for the retrospective study are presented in Table 2. Patients with mixed histologic features, a 13 tumor, or pleural invasion were ineligible. At the completion of the study, the minimal clinical follow-up was 48 months for the last patient included in the cohort. Non-tumoral lymph nodes were obtained after surgery from patients suffering from cardiac diseases. Peripheral blood was obtained from healthy volunteers at the “Centre National de la Transfusion Sanguine” (Paris, France). The protocol was approved by local ethic and human investigations committee (n° 2008-133), and by the Assistance Publique-Hopitaux de Paris (AP-HP), in application with the article L.1121-1 of French law. A written informed consent was obtained from the patients prior to inclusion in the study.

Immunohistochemistry

Serial 5 μm tissue sections of paraffin-embedded lung tumors were deparaffinized, rehydrated, and pretreated in appropriate buffer for antigen retrieval. Then, the sections were incubated with 5% human serum for 30 min before adding the appropriate antibodies or isotype controls. Enzymatic activity was revealed as described previously (MCD, JCO, 2008). When necessary, sections were counterstained with hematoxylin. Images were acquired using a Nikon Eclipse 80i microscope (Nikon, Champigny-sur-Marne, France) operated with Nikon NIS Elements BR software.

Method for Cell Quantification

The cell quantification was measured quantitatively in the tumoral areas of the entire tissue section (original magnification: x100) using the Nikon Eclipse 80i microscope and operated with Nikon NIS Elements BR software. The density of follicular B cells of Ti-BALT was expressed as a surface of follicular CD20+ B cells per tumor intermediate power field (IPF) with SEM calculated. The number of DC-Lamp+ mature DC was lower than the number of cells described above allowing us to realize a quantitative counting (mean DC per tumor IPF with SEM calculated). According to the standard evaluation by pathologists, the necrosis and fibrosis were counted as the percentage of the positive areas among the whole tumor mass section.

Enrichment of Tumor-Infiltrating Lymphocytes

Fresh lung tumor specimens were mechanically dissociated and incubated in a non-enzymatic solution (Cell Recovery solution, BD Biosciences, Le Pont-de-Clai, France) for 1 h at 4°C. The cell suspension was then filtrated through a 70 μm filter (BD Biosciences), and the mono-nuclear cells were isolated by centrifugation over Ficoll Hypaque.

Flow Cytometry

Multiple stainings were performed using antibodies against B cell markers. Briefly, after saturation with 2% human serum, mononuclear cells were incubated with the primary antibodies or appropriate isotype controls for 30 min at 4°C in the dark. Then, cells were washed and fixed in 1% formaldehyde before the analysis on an LSRII cytometer (BD Biosciences). Flow cytometry data were analyzed with the Diva software (BD Biosciences).

Ex Vivo Culture of Tumor-Infiltrating B Cells

Total tumor-infiltrating B cells were isolated from mononuclear cells through a positive selection. Briefly, cells were incubated in presence of anti-CD19 microbeads (Miltenyi Biotech), and then loaded onto a Macs columns (Miltenyi Biotech). Freshly isolated B cells were cultured in presence of Panserin (Staphylococcus aureus cells extract, Calbiochem) or murine CD40 ligand transfected fibroblastic cell line (CD40-L. Cell were kindly provided by Schering-Plough, Laboratory for Immunological Research, Dardilly, France). The supernatant was recovered every 3 days until day 9, and cryopreserved.

Statistical Analysis

Variables taken into account for statistical analysis included clinical (age, sex, smoking, tumor relapse, and vital status), histopathological (histology, pTNM, tumor differentiation, localization of the primary tumor, necrosis, fibrosis, proliferation of the tumor) and immunological parameters (see markers described above). To perform univariate analysis, groups of patients were defined according to the bimodal distribution of the density of positive cells which appointed the following cut-offs. (follicular CD20+B cells: 0.029 mm²/tumor IPF, DC-Lamp: 1.65 mean cells/tumor IPF). Chi-square test with Yates correction and ANOVA test (post-hoc tests with Fisher and Bonferroni methods) were used for univariate analysis. Disease-specific survival (DSS) curve were estimated by Kaplan-Meier method and the significance of differences between groups of patients was evaluated by the Logrank test. An event affecting the OS was defined as death from any cause, DSS as death from NSCLC and DFS as relapse of the primary tumor. Statistical analysis was performed using StatView software. A P value<0.05 was considered statistically significant.

Results

1—Ti-BALT Contain the Same Contingent of Immune Cells as That Observed in Secondary Lymphoid Organs

In order to study the role of the B-cell compartment of Ti-BALT, we first characterized by immunohistochemistry B cells and other immune cells in which they will be in contact in these tertiary lymphoid structures. As described in conventional lymphoid organs, we observed that CD3+ T cells and DC-LAMP+ mature DC cluster to form the T-cell rich areas whereas most CD20+ B cells segregate into B follicles. Within the B-cell areas, different non-B cells were present. We detected few CD3+ T cells which probably represent follicular helper T cells, follicular dendritic cells which are
organized into a network and a specialized population of CD68+ macrophages also called tangible-body macrophages.

[0075] In conclusion, the segregation of immune cells in Ti-BALT is the same as that observed in secondary lymphoid organs, suggesting that immune responses may take place within ectopic lymphoid structures in human lung cancer.

[0076] 2—The B-Cell Areas of Ti-BALT have Features of an Ongoing Humoral Immune Response

[0077] The segregation of T and B-cell areas is a mandatory for the development of both high-affinity class-switched antibodies and memory humoral immune response. Thus, we assessed the stage of B-cell differentiation within the lung tumors, and compared it to secondary lymphoid organs. By immunohistochemistry, we first characterized B-cell subsets according to the Bm classification proposed by Dr. D. Capra (Pasqual et al., J. Exp. Med., 1994). As observed in the secondary follicles of lymph nodes (FIG. 1A), we shown an accumulation of IgD+ naïve B cells in a restricted areas called the mantle (FIG. 1B). This mantle surrounded a germinal centre (GC) as defined by the presence of CD23+ cells which comprised a network of follicular dendritic cells and Bm2 naïve B cells (FIG. 1C-D). GC-B cells were also characterized by the expression of AID (FIG. 1E-F), the enzyme critical for the somatic hypermutation, class switch recombination and gene conversion of immunoglobulin (ig) genes. In a similar manner, GC-B cells were positive for the proliferation marker Ki67 (FIG. 1G-H) and Bcl6 (FIG. 1I-J), but did not express the anti-apoptotic protein Bcl2 (FIG. 1K-L) in Ti-BALT and lymph nodes. Based on the expression of CD138, no plasma cell (PC) was detected in Ti-BALT B follicles (data not shown), in accordance with the situation seen in lymph node (FIG. 1M). However, CD138+ PC were observed in the stroma and the fibrosis of the tumor (FIG. 1N).

[0078] We next further compared the proportion of intra-tumoral B cell subsets to lymph nodes as well as peripheral blood by flow cytometry. According to the expression of IgD and CD38 among total CD19+ cells, the distribution of the five B cell subsets was completely distinct in NSCLC compared to lymph nodes and blood (FIG. 2A). Blood and LN-B cells were mainly of naïve and memory phenotypes (IgD+CD38low naïve B cells, IgD+CD38high memory B cells, respectively). In contrast to blood and lymph nodes, every stage of B cell differentiation was detected in NSCLC. We shown that the percentage of memory B cells and PC was statistically higher, and naïve B cells lower in NSCLC compared to the other two sites (FIG. 2B). 18%, 48%, and 62% of memory cells; 1%, 0.5% and 28% of PC; 75%, 42% and 4% of naïve B cells in blood, lymph nodes and NSCLC. The differential expression of additional B cell markers (CD23, CD27, and CD77) allowed us to study the distribution of subsets of naïve (Bm1 and Bm2), GC (Bm3 and Bm4) and memory (early Bm5 and late Bm5) B cells in NSCLC and lymph nodes. As shown in FIG. 2C, the main difference between the 2 sites was the ratio of Bm1/Bm2 which was in favor of Bm1 in lung tumor. The other ratios analyzed (Bm3/Bm4 and early Bm5/late Bm5) were not statistically different in the 2 anatomic sites.

[0079] All together, these data demonstrate that the differentiation stage of tumor-infiltrating B cells is in accordance with their in situ localization, i.e. in or out of ectopic lymphoid structures. Early differentiated B cells are organized into B follicles of Ti-BALT where the somatic mutation and isotype switching machineries are activated, suggesting that Ti-BALT may be an active site for the generation of memory B cells and antibody-secreting cells.

[0080] 3—Density of Follicular B Cells is Correlated with Long-term Survival, and its Prognostic Value is Enhanced when Associated with the Density of Mature DC

[0081] The presence of reactive B follicles in some lung tumors prompted us to investigate their immunological function. As we have shown that the density of mature DC was associated with a favourable clinical outcome (Diel-Nosjean et al., J. Clin. Oncol., 2008), we investigated whether the densities of follicular B cells and mature DC were correlated to each other as well as their prognostic value. FIG. 3A shows that, even if the global increase of the density of follicular B cells was associated with a global increase of the density of mature DC, these two parameters were not statistically related to each other (R^2=0.1224) suggesting their reciprocal independence. By univariate analysis, we next investigated the prognostic value of follicular B cells alone or in combination with mature DC. The four-year disease-specific survival rates were 97% among patients with high density of follicular B cells (Foll-CD20 High), and 65% among patients with low density of follicular B cells (Foll-CD20 low) (FIG. 3B). Thus, the patients with Foll-CD20 High have a longer survival than patients with Foll-CD20 low (P=0.0099) demonstrating that the number of follicular B cells was associated with a favorable prognosis. There were no distinguishable clinical (sex, age, smoking history), tumor (tumor differentiation, pT/NM staging, fibrosis, necrosis, and proliferating tumor cells), or histologic characteristics between the patients with Foll-CD20 High versus Foll-CD20 low tumors (Table 1). A similar result was obtained for the density of DC-Lamp+ mature DC (FIG. 3C) indicating that both professional antigen-presenting cells were predictive for survival. We next tested whether the patients with “DC-Lamp High” tumors and patients with “Foll-CD20 High” tumors were the same, and vice versa for patients with “DC-Lamp low” tumors and “Foll-CD20 low” tumors. Among the 74 patients, 31 patients (42% of patients) belonged to both groups of “DC-Lamp High” tumors and “Foll-CD20 High” tumors, 17 (23% of patients) belonged to both groups of “DC-Lamp low” tumors and “Foll-CD20 low” tumors, and 26 (35% of patients) were mixed. Among this mix group called “Foll-CD20/DC-Lamp mix”, 21 patients were characterized as having “DC-Lamp High” tumors and “Foll-CD20 low” tumors, and 5 patients with “DC-Lamp low” tumors and “Foll-CD20 High” tumors. The presence of this non-overlapping group is in favor of an independence between these 2 parameters. Due to the limited number of patients within each sub-group of the mix group, we decided to keep the 26 patients within a unique group. The Kaplan-Meier curves indicated that 100% of patients with “Foll-CD20/DC-Lamp high” were alive (none event among the 31 patients) after a follow-up of 48 months (FIG. 3D). We found that patients with low density of both intra-tumoral mature DC and follicular B cells (“Foll-CD20/DC-Lamp low” group) had a very poor prognosis with 38% of surviving patients after 48 months (6 events out of 17 patients). The survival curves of patients with “Foll-CD20/DC-Lamp mix” tumors were between these two curves with 86% of alive patients (3 events out of 26 patients). The median disease-specific survival was only reached for the patients with “Foll-CD20/DC-Lamp low” tumors (42 months, P=0.0099).

[0082] In conclusion, we demonstrated that the density of tumor-infiltrating follicular B cells is highly predictive of
disease-specific survival in early-stage NSCLC. Compared to the density of each cell type, the combination of both mature DC and follicular B cells allows the identification of a group of patients without any event and a group with most events.

Example 2

**[0083]** 1—Prognostic Value of Follicular B Cells and of Mature Dendritic Cells in Patients Treated by Neo-Adjuvant Chemotherapy

**[0084]** The five-year survival of patients with early-stage NSCLC is 70%, and drops to 15% for late-stage metastatic NSCLC. Studies have shown that two-drug combinations are more efficacious than single-agent treatment (Schiller et al., 2000). Presently, patients with advanced NSCLC receive a neo-adjuvant polychemotherapy (cisplatin plus gemcitabine or carboplatin plus paclitaxel) in many North American and European hospitals (Bunn et al., 2002; Rosell et al., 2002). Now, more and more patients with early-stage lung cancer also receive neo-adjuvant chemotherapy. The response rate of two-drug combinations is between 20 to 30% in advanced NSCLC. Recent reports indicate that cytotoxic drugs are not only targeting tumor cells, but can indirectly promote tumor control by facilitating the development of an immune response within the tumor microenvironment (reviewed in Zitvogel et al., 2008).

**[0085]** Lung cancer contains tumor cells as well as stroma components: the vasculature, connective tissue and immune infiltrating cells. Among immune cell infiltrate, some of them are professionals for the presentation of processed antigens, like DC, B cells and macrophages. Recently, we have shown that the density of different DC subsets (epithelial Langerhans cells, stromal interstitial DC and mature DC) was associated with a favorable outcome in NSCLC patients (Dieu-Nosjean et al., 2008; Fridman et al., 2011). Now, we show that the density of mature DC is still correlated with a longer-term survival for advanced NSCLC patients treated by neo-adjuvant chemotherapy (n=56 patients, P=0.0373, see FIG. 4).

**[0086]** As Ti-BALT are present in patients treated by chemotherapy, the density of B cells, another antigen-presenting cell population, can be associated with the outcome of patients treated by chemotherapy. The density of B cells may also be impacted by the type of the two-drug combinations. Thus, the B-cell marker will be correlated to clinical response to treatment. This biomarker can be used to discriminate between patients who will respond and patients who will not respond to the treatment, and thus allowing a more rational and targeted therapy design.

Example 3

**[0087]** Here, we evaluated the prognostic value of either follicular B cells, mature DC, or the combination of both types of immune cells in a retrospective study of 122 patients with advanced-stage of NSCLC and treated by neo-adjuvant chemotherapy. We demonstrated that the density of each immune parameter was correlated with a favorable outcome. The Kaplan-Meier curves indicated that the density of CD20+ follicular B cells was associated with longer overall survival (OS, P=0.007) (FIG. 5A). The median OS was 55 months for the patients characterized as having Foll-CD20 High tumors whereas the median OS was 18 months for patients with Foll-CD20 low tumors. The density of DC-Lamp+ mature DC was also correlated with a better clinical outcome (FIG. 5B, P=0.04). The median OS was 55 months for the patients with DC-Lamp High tumors whereas the median OS was 24 months for patients with DC-Lamp low tumors.

**[0088]** As Foll-CD20 and DC-Lamp positively influence the patient survival, we stratified the patients into 3 groups according to the high/low densities of each marker (Foll-CD20/DC-Lamp High, Foll-CD20/DC-Lamp mix, and Foll-CD20/DC-Lamp low). Patients with high density of both types of immune populations were at low risk of death (median OS was not reached) (FIG. 5C, P=0.005). Patients with low densities of both follicular B cells and mature DC were at very high risk of death (median OS was 18 months), demonstrating that the combination of both immune markers allows the identification of a subgroup of patients with a very poor outcome despite chemotherapy. Patients with “Foll-CD20/DC-Lamp mix” tumors were at intermediate risk of death (median OS was 34 months).

**[0089]** These data were in accordance with univariate analyses (Table 3) showing that the density of each immune cell type is highly associated with the survival of patients treated by chemotherapy (Foll-CD20 with a Hazard Ratio (HR) =0.41 and 0=0.00095; DC-Lamp with a HR=0.50 and P=0.00721). More interestingly, the combination of both biomarkers was the better predictor for survival (HR=0.50, P=0.000097) compared to standard clinical parameters.

**[0090]** All together, we have demonstrated that the combination of CD20 and DC-Lamp markers is able to discriminate patients with very high risk of death among advanced-stage NSCLC patients treated by neo-adjuvant chemotherapy.

REFERENCES

**[0091]** Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.


1. An in vitro method for the prognosis of survival time of a patient suffering from a solid cancer, comprising the following steps:
   a) quantifying on the whole tumor, the cell density of follicular B cells, and
   b) comparing the cell density of follicular B cells value obtained at step a) with a predetermined reference value; and
   c) providing a favorable prognosis of survival time for said patient when the cell density of follicular B cells is higher than said predetermined reference value, or providing a poor prognosis of survival time for said patient when the cell density of follicular B cells is lower than said predetermined reference value.

2. The method of claim 1 wherein the survival is the disease-specific survival (DSS), the disease-free survival (DFS) or the overall survival (OS).

3. The method according to claim 1 further comprising the steps of:
   a) quantifying on the whole tumor, the cell density of mature dendritic cells, and
   b) comparing the cell density of mature dendritic cells value obtained at step a) with a predetermined reference value; and
   c) providing a favorable prognosis of survival time for said patient when the cell densities of both the follicular B cells and of the mature dendritic cells are higher than said predetermined reference values, providing a poor prognosis of survival time for said patient when the cell densities of both follicular B cells and of the mature dendritic cells are lower than said predetermined reference values, or providing an intermediate prognosis of survival time for said patient when one value of the cell densities of follicular B cells and of the mature dendritic cells is lower than said predetermined reference value and the other value is higher than said other predetermined reference value.

4. The method of claim 1 wherein the solid cancer is selected from the group consisting of lung cancers, colorectal cancers and breast cancers.

5. The method of claim 1 wherein the solid cancer is a lung cancer.

6. The method of claim 1 wherein the solid cancer is a non-small cell lung cancer.

7. The method of claim 1 wherein the patient is a patient with an early stage of cancer.

8. The method of claim 1 wherein the patient is a patient with early stage of cancer who did not receive any neoadjuvant or adjuvant therapy.

9. The method of claim 1 wherein the patient is a patient with advanced stage of cancer.

10. The method of claim 1 wherein, the patient is a patient with advanced stage of cancer who receives adjuvant therapy.

11. The method of claim 1, further comprising the step of, if said patient has a poor prognosis of survival time then providing aggressive treatment for said patient, but if said patient has a favorable prognosis of survival time then not providing aggressive treatment for said patient.

12. The method of claim 1, wherein said step of quantifying is carried out by contacting follicular B cells with an antibody against CD20 and analyzing the follicular B cells by flow cytometry.

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