

## RESEARCH ARTICLE

## Diagnostic Value of Interleukin 21 and Carcinoembryonic Antigen Levels in Malignant Pleural Effusions

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### Abstract

The aim of this study was to evaluate the diagnostic value of interleukin 21 (IL-21) and carcinoembryonic antigen (CEA) in tuberculous pleural effusions (TPEs) and malignant pleural effusions (MPEs). Pleural effusion samples from 103 patients were classified on the basis of diagnosis as TPE (n=51) and MPE (n=52). The concentration of IL-21 was determined by ELISA. Lactate dehydrogenase (LDH), adenosine dehydrogenase (ADA) and CEA levels were also determined in all patients. A significant difference was observed in the levels of ADA and CEA ( $P < 0.01$ ), but not in the levels of LDH ( $P > 0.05$ ) between TPE and MPE. The concentration of IL-21 in MPE was significantly higher compared to TPE ( $P < 0.01$ ). With a threshold value of 4.32 pg/ml, IL-21 had a sensitivity of 76.9% (40/52) and a specificity of 80.4% (41/51). Combined detection of IL-21 and CEA had a sensitivity of 69.2% (36/52) and a specificity of 92.2% (47/51). These two markers can contribute to the differential diagnosis of MPEs.

**Keywords:** Interleukin 21 - carcinoembryonic antigen - malignant pleural effusion - tuberculous pleural effusion

*Asian Pacific J Cancer Prev*, 13, 3495-3499

### Introduction

Pleural effusion is one of the most common manifestations of pleural involvement in many diseases. In healthy people the pleural cavity, between the parietal and visceral pleural, contains little amount of fluid that plays mainly a lubricating role. This fluid is continuously filtered out and absorbed in a physiological dynamic balance. During intra- and extra pulmonary diseases, this balance is disturbed leading to either a decrease in absorption or increase in filtration, thus increasing the amount of fluid termed as pleural effusion (Doelken, 2008; Khaleeq et al., 2008; Musani, 2009).

Pleural effusions can be divided into benign and malignant. The most common causes of benign pleural effusion are pneumonia and tuberculosis, whereas lung and breast cancer account for the majority of cases of malignant pleural effusions (MPE) (Spector et al., 2008; Heffner, 2008). Differentiating between these two types in clinical practice is essential, but remains a major diagnostic challenge. Pleural fluid cytology has traditionally been the analytical method of choice for the detection of tumor cells in pleural fluid. However, sensitivity varies between 30% and 60% and the method is thus far from perfect (Sriram et al., 2011). In negative cytology samples, although detection of tumour markers like carcinoembryonic antigen (CEA), adenosine dehydrogenase (ADA) and

lactate dehydrogenase (LDH) by immunohistochemistry can be performed, sensitivity and specificity remain limited (Radjenovic-Petrovic et al., 2009; Gupta et al., 2010; Hackbarth et al., 2010). Moreover, the sensitivity and specificity of immunohistochemistry are affected by the levels of expression of the protein being evaluated, antibody specificity and the quality of the pleural fluid sample provided to the laboratory (Pomjanski et al., 2005; Westfall et al., 2010). When cytology in conjuncture with immunohistochemistry does not provide a definitive diagnosis, more invasive procedures are indicated. While pleural biopsy yields a high sensitivity (Maskell et al., 2003; Casal et al., 2009), the rate of false negative is between 5-15% and the risk of complications associated with thoracoscopy are non-negligible (Davies et al., 2010). Therefore the search for novel indices is important in order to make up for the limitations of pleural cytology and immunohistochemistry, and to obviate the need for invasive procedures.

Cytokines are secreted signalling molecules with decisive effects on haematopoiesis, innate and adaptive immunity, and immunopathology. Interleukin 21 (IL-21) is a four-helix bundle cytokine that is produced by activated CD4+ T cells and natural killer (NK) T cells (Søndergaard et al., 2009). This novel cytokine was initially discovered by functional cloning after expression of the IL-21 receptor (IL-21R) gamma chain in BaF3 cells and a library

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from activated T cells to screen for ligands (Parrish-Novak et al., 2000; Harada et al., 2006; Coquet et al., 2007). The IL-21 receptor (IL-21R) is expressed on the surface of T, B and NK cells. IL-21r is similar in structure to the receptors for other type I cytokines like IL-2R (Ozaki et al., 2000) or IL-15 and requires dimerization with the common gamma chain ( $\gamma_c$ ) in order to bind IL-21 (Asao et al., 2001; Habib et al., 2002). When bound to IL-21, the IL-21 receptor acts through the Jak/STAT pathway, utilizing Jak1 and Jak3 and a STAT3 homodimer to activate its target genes (Ozaki et al., 2000).

IL-21 has pleiotropic effects on both innate and adaptive immune responses. These actions include positive effects such as enhanced proliferation of lymphoid cells, increased cytotoxicity of CD8+ T cells and NK cells, and differentiation of B cells into plasma cells. Conversely, IL-21 can also be proapoptotic for B cells and NK cells. IL-21 is also produced by Th-17 cells which are a distinct subset of T helper cells, and is a critical regulator of Th-17 development (Spolski et al., 2008). However, while Th-17 has been found to be increased in tuberculous pleural effusion (TPE) and MPE, no studies till date have reported whether IL-21 is also involved.

The aims of this study were firstly to investigate the activity of IL-21 in TPE and MPE, and its potential in differentiating between these two conditions.

## Materials and Methods

In this study pleural effusion samples were collected from 103 patients who were hospitalized in the Department of Respiratory Medicine, Tongji Hospital Affiliated to Tongji Medical College, Huazhong University of Science and Technology. Care was taken to make sure that the patients matched the diagnostic criterias and didn't suffer from chronic diseases such as chronic viral infections (Elsaesser et al., 2009), SLE (Sarraf et al., 2010), Rheumatoid Arthritis (Daha et al., 2009), Psoriasis (Liu et al., 2008) and Inflammatory bowel diseases (Liu et al., 2009). Written consent was obtained from all the patients concerned in order to perform this study. Pleural effusions were divided into TPE (51 cases; 37 males and 14 females; 39.48±3.838 years of age) and MPE groups (52 cases; 38 males and 14 females; 58.41±2.038 years of age).

### Diagnostic criteria for pleural effusions

Using Lights criteria the pleural effusions were firstly divided into transudate and exudate. TPE was diagnosed according to the following principle: identification of M. tuberculosis, pleural biopsy revealing granulomatous tissue, positive PPD test, positive response to anti-Tuberculous treatment and high levels of ADA. The diagnostic criteria for MPE were: Cytological evidence of malignant cells present in pleural effusion, high CEA values or from tissue biopsies taken.

### Samples collection

Pleural effusions were collected before any treatment was initiated within 24 hours of hospitalization. Some of the pleural fluids were sent to the hospital laboratory to detect levels of total protein (Pro), LDH, ADA and CEA.

Some other pleural effusions (100 ml) were centrifuged at 4 °C 1200 r/min for 15 min, and the supernatants were immediately frozen with 500 µl Ep tubes at -80 °C.

### Measurement of IL-21

The concentration of IL-21 in pleural effusions was measured by the enzyme-linked immunosorbent assay kit (ELISA) according to the manufacturer's protocol. All samples were assayed in duplicate.

### Statistical Analysis

Data were expressed as the means ± SEM. Difference in data was analyzed by the Student's t-test or the  $\chi^2$  test, using receiver operating curve (ROC) analysis to evaluate the threshold value of IL-21 and CEA in differentiating TPE from MPE. For each ROC, a cut-off point was determined as the value of the parameter that maximized the sum of specificity and sensitivity. A value of P<0.05 was considered significant. Statistical analysis was carried out using SPSS 17.0 software.

## Results

### General characteristic of the pleural effusions

Significant differences were observed in age, as well as CEA, Pro and ADA levels in MPE and TPE (P<0.05). We did not find a significant difference in the concentration of LDH between MPE and TPE (P>0.05).

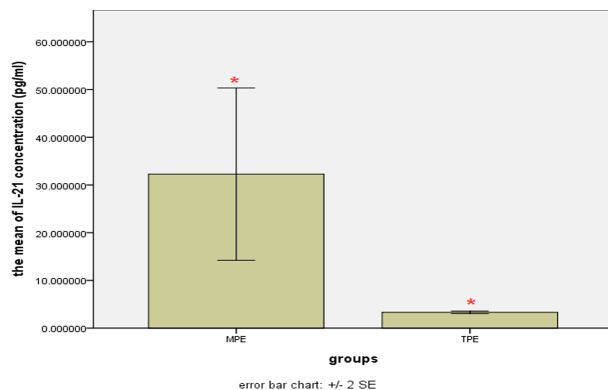
### IL-21 concentration in pleural effusions

As shown in Figure 1, the concentration of IL-21 in the MPE group (32.27±9.027 pg/ml) was significantly higher compared to the TPE group (3.32±0.136 pg/ml; P<0.01). Diagnostic value of IL-21 in TPE and MPE.

The diagnostic threshold afforded by the ROC analysis for IL-21 was 4.32pg/ml. The area under the IL-21 ROC was 0.98. Using a threshold value of 4.32 pg/ml, IL-21 had a sensitivity of 76.92% (40/52), a specificity of 80.39% (41/51).

### Diagnostic value of combined detection of IL-21 and CEA in TPE and MPE

Firstly, CEA levels were detected and the diagnostic value in TPE and MPE was analyzed. The diagnostic threshold afforded by the ROC analysis for CEA was



**Figure 1. Concentration of IL-21 in MPE and TPE.** Data are presented as the means ± 2\*SEM and the value between the MPE and TPE groups differs significantly. \*P<0.01

3.48ng/ml. The area under the CEA ROC was 0.967. It was lower compared to the areas of IL-21. With a threshold value of 3.48ng/ml, CEA had a sensitivity of 75% (39/52), a specificity of 86.27% (44/51). The sensitivity of IL-21 was higher compared to CEA. Between the studied parameters, IL-21 and CEA, no significant differences were found with respect to the specificity. The combined diagnostic value of the IL-21 and CEA in TPE and MPE was further analysed. The results showed that the combined detection of these two indices had a sensitivity of 69.23% (36/52) and a specificity of 92.16% (47/51). The specificity was higher compared to the separate tests for TPE and MPE. However, no significant differences were found with respect to sensitivity.

## Discussion

This study is the first to investigate the potential of IL-21 as a possible marker in differentiating between benign and malignant pleural effusions. We show that the level of IL-21 is higher in MPE than in TPE. The level of CEA was also significantly higher in MPE. We also investigated other indices such as LDH and Pro, and there were no significant differences in their concentration in TPE and MPE. Furthermore, when using the combined detection of IL-21 and CEA the diagnostic value was much more accurate.

IL-21 is expressed on activated CD4+ T cell and is involved with interactions between B cells, T cells, NK cells, and dendritic cells. It may thus be considered as a key cytokine linking innate and adaptive immune responses. IL-21 was shown to play a key role in T helper (Th) cell differentiation. Th cells can be divided into three distinct lineages: Th1 cells are involved in supporting CD8+ effector T-cell function; Th2 cells tend to promote antibody responses; and the recently described Th-17 cells promote inflammatory responses and play an important role in autoimmunity (Hoeve et al., 2006; Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007). IL-21 induces maturation and activation of NK and NKT cells and thereby increases the cytolytic potential of NK cells (Brady et al., 2004), with induction of various genes involved in activation of innate immune responses. Current data also suggest that IL-21 has a complex role in regulating B cell differentiation, proliferation and immunoglobulin production. IL-21 has also been shown to possess pro-inflammatory functions on connective tissue cells such as fibroblasts, indicating that it may be an important mediator in autoimmune diseases (Brandt et al., 2007). In view of its wide range of actions on both immune and non-immune cells, IL-21 may play an important role in the pathogenesis of some diseases.

The main cellular components of both tuberculous and malignant pleural effusions are lymphocytes (Lucivero et al., 1988). Large scale studies have reported that CD4+ T lymphocytes play an important role in the pathogenesis of pleural effusion (Yang et al., 2008). Recently, Th-17 cells have been found to be increased in pleural effusion of tuberculous and malignant origins (Wang et al., 2011; Ye et al., 2011). It has been reported that IL-21, TGF- $\beta$ , IL-1 $\beta$  and IL-6 are the main inducers of Th-17 differentiation

(Acosta-Rodriguez et al., 2007). Th-17 cells have been proposed to play important roles in several human diseases, including autoimmune conditions, allergy, the development and progression of tumours, and bacterial and fungal infections (Chen et al., 2008). Th-17 selectively produces proinflammatory cytokines including IL-17, IL-21, and IL-22 (Spolski et al., 2008). Our study is the first to compare the levels of IL-21 in these two types of effusion.

Tuberculosis is a common cause of benign pleural effusions and TB-related pleural effusions can occur in approximately 2-10% of patients with TB (Baumann et al., 2007). T cell response is critical in the protective immunity against *Mycobacterium tuberculosis*. More recently, Th-17 has been associated with *M. tuberculosis* infection and significant increases of this subset of T cell have been found in TPE (Wang et al., 2011). However, although IL-21 is produced and regulated by Th-17, in our study IL-21 is not significantly upregulated in TPE. This is an unexpected finding which suggests different pathways exist for Th-17 activation. Increased levels IL-1 $\beta$  and IL-6 have been found in TPE, but with low levels of TGF- $\beta$  (Wang et al., 2011). Also in IL-6 deficient mice, Th-17 differentiation is driven by the synergistic action of IL-21 and TGF- $\beta$  (Korn et al., 2007). Similarly in mice immunized with TB vaccines, both IL-21 and TGF- $\beta$  were increased in post challenge test (Lim et al., 2009). It is therefore possible that in TPE, IL-21 does not primarily drive Th-17 differentiation and IL-6 is the more important cytokine. Further studies are required to define the precise role of IL-21 in mediating immunity against *M. tuberculosis*.

In this study, the concentration of IL-21 was much higher in MPE than TPE, indicating the involvement of IL-21 in the pathogenesis of malignant pleural effusions. Considering the profound effects of IL-21 on T and NK cells and the proven antitumor effects of another gamma-chain cytokine (IL-2), it is not surprising that IL-21 has antitumor effects (Wang et al., 2003). These have been observed in animal models including melanoma, sarcoma, and bladder and renal cell carcinoma (Ma et al., 2003; Furukawa et al., 2006; Søndergaard et al., 2007). Through depletion studies, the antitumor activity of IL-21 was shown to depend on NK cells or CD8+ T cells, or both, depending on the tumor model. Thus, while IL-21 immunotherapy has been investigated, little is known regarding its function in the tumorigenesis. Other studies concerning MPE have shown that Th-17 and the cytokines needed for Th-17 differentiation such as TGF- $\beta$ , IL-1 $\beta$  and IL-6 were all increased (Ye et al., 2011). Again, we can only put forward the hypothesis that the increase in IL-21 in MPE might be related to that of TGF- $\beta$ , although the precise mechanism remains unknown. Nonetheless, given the increasing use of IL-21 as antitumor therapy in various cancers, further studies are needed not only for its use as tumor marker, but also as potential treatment option for MPE.

CEA is one of the most widely studied and recommended tumour markers and has been found to have a good performance at diagnosing MPE. CEA levels may increase in pleural fluid following obstruction to lymphatic drainage by malignant cells and pleural invasion (Ferrer et

al., 1999). It has been reported that CEA concentration in malignant tissues was on average 60-fold higher compared to the non-malignant tissues (Boucher et al., 1989). In our study the diagnostic value of CEA in MPE and TPE was 3.48ng/ml and had a sensitivity of 75% (39/52), a specificity of 86.27% (44/51). Using a threshold value of 4.32pg/ml, IL-21 had a sensitivity of 76.92% (40/52), a specificity of 80.39% (41/51).

The combined diagnostic value of the IL-21 and CEA in TPE and MPE was further analysed. The results showed that the combined detection of these two indices had a sensitivity of 69.23% (36/52) and a specificity of 92.16% (47/51). The specificity was higher compared to the separate tests for TPE and MPE. This shows that the combined use of IL-21 and CEA had a better diagnostic value than the use of a single index. This may provide a new approach in the differential diagnosis of MPE and TPE.

In summary, our results suggest that IL-21 is higher in MPE compared to TPE. The critical value of IL-21 protein for diagnosing MPE and TPE was 4.32 pg/ml. The combined detection of CEA and IL-21 had a higher specificity compared to the single index, which effectively improves the diagnostic significance of IL-21. However further studies are required to examine the exact role of this cytokine in the etiopathogenesis of MPE.

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