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Investigating new serological and tissue markers for the follow-up of patients operated for alveolar echinococcosis.

Running head: new biomarkers for operated AE patients

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Author contributions:

APB, CR, SBH, CT and LM included the AE operated patients from the french center (Besancon) and looked for their clinical characteristics. BG, JW, AL and GB included the AE operated patients from the french center (Besancon) and looked for their clinical characteristics.
HH performed the statistical analysis. ZI performed the microarray analysis. CB performed the serology analysis. APB, LM, BG, AL and GB wrote the manuscript and each gave their expertise on AE field and immunology aspects.

Abstract

Aims: Alveolar echinococcosis (AE) is characterized by a chronically progressing hepatic injury caused by *Echinococcus multilocularis.* Surgery presently remains the best curative option. Currently, biological predictive features derived from the resected specimens are not suitable to assess surgery efficacy. The present study was designed to investigate whether a selection of markers measured on the resected specimens exhibits predictive features related to parasite viability, or to a total elimination of the parasite, in addition to serological markers.

Methods and results: In a collaboration between two centers, one in France (Besançon), and one in Switzerland (Bern), samples from 40 AE patients were analyzed by microarray and serology techniques, individually. Paired serum samples before and after surgery were obtained for 26 patients. In the sera, a significant decrease of PD-L1 levels was observed after surgery, in addition to anti-Em18 levels. In the liver tissue, low levels of Cluster of Differentiation (CD)-3 were correlated with the absence of serum anti-Em18 after surgery.

Conclusion: This study showed PD-L1 is promising as a potential serological marker, and further confirmed the performance of anti-Em18 serology. Further studies on a larger cohort is needed to confirm the utility of performing systematically microarray on resected liver tissue.

Key words: follow-up, serology, microarray, PD-L1, Em18, CD-3

Introduction

Alveolar echinococcosis (AE) is a severe chronic helminthic human disease, caused by *Echinococcus multilocularis* (*E.multilocularis*). AE exhibits morphologically and clinically a tumour-like pattern, as the parasite progressively growths mostly in the liver, with a subsequent potential to extend to adjacent organs by contiguous progression and to spread to distant organs *via* blood circulation leading to metastases.

Nowadays, thanks to generalization of imaging techniques, AE cases are more and more incidentally diagnosed at early stages.[1] Increasing incidences of AE in European countries such as Switzerland, France, Germany and Austria were reported recently.[2] Cases of AE were also described in patients receiving immunosuppressive therapy for malignant and inflammatory diseases; these patients are presumably at increased risk for occurrence, delayed diagnosis and progression of AE.[3]

Radical surgery is the only curative option for the treatment of AE, especially when the complete resection (R0) is possible. Partial resections (R2) are more associated with recurrences and are thus less and less performed.[4] Currently, no good biomarkers exist that may predict risk of recurrence after a presumable curative surgery. Follow-up after surgery currently includes i) medical therapy using mostly albendazole for 2 years, ii) monitoring by imaging and iii) annual serology follow-up, using primarily recEm18-ELISA.[5,6] Currently, 10 years follow-up after curative surgery is recommended because of the risk of recurrence.[4,7]

Current options that are currently available for follow-up are mainly recEm18-ELISA that has shown predictive values.[5,6] Indeed, upon complete surgical removal of the parasite, the decrease of Em18 antibody levels in serum was shown to be early and significant.[6,8] Thus, recEm18-ELISA in combination with imaging, proved to be optimal to monitor AE patients.[6]

In the context of AE management after surgery, the availability of biomarkers predictive of parasitic activity could be helpful for the patients follow-up. However, no predictive biomarkers from the resected liver tissue are available. This type of information could be of help to decide if and how long the adjuvant treatment of albendazole is needed. Therefore, we tested the hypothesis

that immunological biomarkers in the serum and resected liver tissue may predict the outcome. Given that the impact of the immune system on controlling the disease and the observation that clusters of differentiation (CD), previously shown to be concentrated in the human liver tissues during AE, such as CD-20, CD-38 and CD-68, were included in the tissue microarray panel.[9] Given the recent findings that inhibitory immune checkpoints are involved in the immune response during alveolar echinococcosis, a focus was made on the programmed death-1 (PD-1)/PD-ligand 1 (PD-L1) signaling pathway, measured in sera and liver tissue.[10-12]

Material and Methods

Samples and clinical data

Two centers, one in France (Besançon), and one in Switzerland (Bern), collected sera, biopsy and clinical data from AE patients who underwent surgery. Two serum samples per patients were collected, one before surgery when available, and one at follow-up, up to several years after. Diagnosis was based on Em2 ELISA and imaging. The PNM classification and staging of alveolar echinococcosis (AE), according to WHO-Informal Working Group on Echinococcosis was used to investigate the gravity: P stands for hepatic localization of the parasite, N stands for extra hepatic involvement of neighboring organs and M stands for the absence or presence of distant metastasis.[13] The surgery was classified as complete "R0" if the margin was ≥ 1mm, and "R1" if <1mm.[7] The criteria "immunosupression" was applied for patients under immunosuppressive therapy such as steroids, methotrexate or rituximab.[3] The clinical data collected included: date of diagnosis, date of surgery, type of surgery (R0/R1), being symptomatic at diagnosis, PNM staging, duration of albendazole treatment, immunosuppression criteria, evolution.

Serology assays

The recEm18-ELISA assays were carried out, as described previously.[5,14] The serum levels of anti-Em18 antibodies were compared before and after surgery. The PD-1 and PD-L1 dosages (Human Platinum ELISA, Fisher Scientific, Illkirch, France) were performed on the collected sera according to the manufacturer instructions. The serum levels of PD-1 and PD-L1 were compared before and after surgery.

ngTMA® construction

All FFPE blocks were cut at 2.5 µm and freshly stained with hematoxylin and eosin (HE). Tissue microarray construction was performed using a next-generation tissue microarray (ngTMA®) approach at Institute of Pathology – University of Bern (Switzerland).[15]

HE slides were scanned using a Pannoramic P250 scanner (3DHistech, Hungary). Next, digital slides were annotated using a TMA tool (Pannoramic Viewer, 3DHistech, Hungary). The digital slides of each surgical resection were annotated using a 1.0-mm tool. These annotated core regions

were punched out using an automated tissue microarrayer, TMA Grandmaster (3DHistech, Hungary).

Immunohistochemistry

Immunohistochemical (IHC) staining was performed using an automated system (BOND RX, Leica Biosystems, UK). Briefly, TMA blocks were cut at 2.5 µm, deparaffinized, and rehydrated in dewax solution (Leica Biosystems). All samples were incubated with the following primary antibodies for 30 min at room temperature: CD-3 (Abcam, SP7 clone, 1:400); CD-4 (Novocastra, 4B12 clone, 1:100); CD-8 (Cell Marque, C8/144B clone, 1.100); CD-20 (Agilent , L26 clone, 1.100); CD-38 (Novocastra, SPC32 clone, dilution 1:100); CD-56 (Novocastra, TB01 clone, 1:100); CD-68 (Agilent, KP1 clone, 1:5000); FoxP3 (ebioscience, 1:200); PD-1 (Cell marque, NAT105 clone, 1:100) and PD-L1 (Cell Signaling, E1L3N clone, 1:400); Tris buffer (pH 9) at 95°C for 30 min was used for antigen retrieval.

Antibody detection was performed with Bond Polymer Refine Detection kit (Leica Biosystems, DS9800) using 3,3-diaminobenzidine (DAB) as brown chromogen and following the manufacturer's instructions. The samples were counterstained with hematoxylin, dehydrated and mounted with Pertex (Sakura). Slides were scanned on Pannoramic 250 Flash (3DHISTECH).

Statistical analysis

For comparison of data, two different analysis were performed. First, a Wilcoxon signed rank test for paired data compared the evolution of anti-Em18, PD-1 and PD-L1 in serum before and after surgery. Secondly, we explored the relationship between each markers measured in the resected tissue and anti-Em18 levels using a Spearman test. A p value < 0.05 was considered as statistically significant. Statistical analysis was carried out using the software Stata v14.1.

Results

Patients

A total of 40 patients that underwent surgery for AE, diagnosed between 2004 and 2016, were included: 14 from the French center (Besançon), and 26 from the Swiss center (Bern). Characteristics of patients, from each center, are listed in Table 1. The median age was similar between the two centers (56 year-old in Bern (Switzerland) *vs* 61 year-old in Besancon (France) (Table 1). The Swiss cohort included much more immunocompromised patients than the French one (38% *vs* 7%, Table 1); these patients presented with either cancer, rheumatoid arthritis, lupus, allergic asthma, or viral infection (hepatitis, herpes). Only three of these patients had immunosuppressive therapy before surgery (cortisone, prednisone) (Table 1). The primary size of lesions was similar for the patients of both centers (4.5 [0-20]) (Table 1). Complete resection (R0) was achieved in 88% of the Swiss patients compared to 50% in the French series (Table 1). No recurrence and no mortality were reported. None of the patients had persistent lesions by imaging. We distinguished those who displayed a total abrogation of parasitic activity (negative anti-Em18 levels) from those with persistent signs of residual parasitic activity (positive anti-Em18 levels). In the Swiss cohort, 47% had persistent signs of residual parasitic activity based on positive anti-Em18 levels. In the French cohort, 18% had persistent signs of residual parasitic activity based on positive anti-Em18 levels (Figure 1).

Paired serology results

The serologic markers studied were anti-Em18, PD-1 and PD-L1. A serum before surgery was available in the serum bank for 26 patients. The baseline sera ("S1") were collected with a median of 3.5 months before surgery [from D0 to 3 years]. The 2nd sera ("S2") were collected up to several years after surgery, with a median of 3 years after surgery [from 7 months to 11 years]. Figure 2 represents the paired variations of anti-Em18, PD-1 and PD-L1 levels, before and after surgery, for 26 patients. A significant decrease for serum anti-Em18 and PD-L1 levels was observed before and after surgery but not for PD-1 levels (Figure 2ABC). A total of seven patients had no anti-Em18 levels detectable either before or after surgery (27%), ten patients with positive

anti-Em18 levels before surgery had no anti-Em18 levels detectable after surgery (38.5%), and nine patients had positive anti-Em18 levels both before and after surgery (34.5%) (Figure 2A). The relative decline of anti-Em18 levels in percentage (whose calculation was [(S1anti-Em18-S2anti-Em18)x100] / S1anti-Em18] was in median 87% [12.5-98].

Concerning PD-L1 levels and their evolution (Figure 2B): the median level of PD-L1 before surgery was 24.98 ng/mL [0-106.33] and the median level of PD-L1 after surgery was 10.64 ng/mL [0-55.17]. The median reduction of the PD-L1 levels was 9.18 with an interquartile range (IQR) of 1.98 to 17.51. Two patients had no PD-L1 detectable before surgery (7.7%), 4 patients with positive PD-L1 levels before surgery had no PD-L1 detectable after surgery (15.4%), and 20 patients had positive PD-L1 levels before and after surgery (77%). The median relative decline of PD-L1 in percentage (whose calculation was [(S1PD-L1-S2PD-L1)x100] / S1PD-L1] was 45% [4-97.5]. The three immunocompromised patients taking immunosuppressive therapy before surgery had barely detectable levels of PD-L1.

Relationship between Em18 and microarray markers

Given that levels of anti-Em18 antibodies is the recognized gold standard to assess parasite activity and viability, a correlation between the absence of detection of anti-Em18 levels after surgery and microarray markers was performed. A significant correlation between absence of detection of anti-Em18 levels after surgery and lower levels of CD-3 (40.4 ± 11.1 *vs* 51.8 ± 14, *p* 0.02) measured by microarray analysis on liver resected tissue was observed (Figure 3). No other significant correlation with anti-Em18 for any other markers measured by microarray was observed (Figure 3).

Discussion

In search of biomarkers useful for the follow-up of AE patients who underwent surgery, microarray on resected liver tissue was performed as well as anti-Em18, PD-1 and PD-L1 measurements in sera. Serology assays showed a significant decrease of anti-Em18 and PD-L1 levels after surgery. The value of anti-Em18 for the follow-up of AE patients was largely demonstrated and is well recognized.[5-6,8] The programmed death-1 (PD-1)/PD-ligand 1 (PD-L1) signaling pathway is a negative regulatory mechanism that inhibits T cell proliferation and cytokine production. Soluble PD-1 and PD-L1 levels in cystic echinococcosis were shown to decrease significantly after radical surgery and albendazole treatment.[10]

The PD-1/PD-L1-pathway was also shown to be overexpressed at the chronic stage of AE and its blockade limited AE development in mice.[10-12;16] This study showed that serologic levels of PD-L1 significantly decrease after liver resection in AE patients who underwent surgery, with a median reduction of 9.2 ng/mL (IQR: 1.98-17.51). Our results suggest that immunosuppressive therapy could affect the detection of PD-L1, but this need to be confirmed on a greater number of patients (only three in this study with immunosuppressive therapy).

Compared to serology assays, microarray techniques are much more expensive and require specialized laboratories. We used here the microarray technology to measure a whole batch of immune biomarkers *in situ* in the resected liver tissue, which is precious and innovative data.

In this study, most patients underwent a complete resection, and no recurrence was subsequently observed, even in immunosuppressed patients. Among the 26 patients for whom sera were obtained before and after surgery, only 19 had a variation of anti-Em18 levels between these two time points (7 patients with anti-Em18 = 0 from the start). Analysis of microarray results showed that lower levels of CD-3 in the resected liver tissue, a recognized marker of T-cells activation, was the only marker associated with absence of detection of anti-Em18 after surgery. Low levels of CD-3 in liver biopsies combined with metastatic pattern by imagery (small or barely visible lesions) could possibly become a tool in the future to dispense initially with therapy. In further

studies, it could also be interesting to follow closely the variation of lymphocytes counts according to the level of CD-3 in the resected tissue and the reduction of PD-L1 levels after surgery.

This study has limitations; one being its retrospective aspect and the fact that both centers differed on some points: classification based on imagery, type of imagery used, rhythm of consultations for follow-up for example. Another limitation was the low number of patients with serum available before and after surgery.

Similar assays on a greater number of AE patients who underwent surgery is needed to confirm that no other markers tested in this study is contributive to better anticipate the persistence of parasitic activity.

Conclusion

This study showed PD-L1 is promising as a potential serological marker, and further confirmed that anti-Em18 serology was the most appropriate biomarker for the follow-up of AE patients who underwent surgery. Similar studies on a greater number of AE patients who underwent surgery are needed to confirm the utility of performing microarray analysis systematically on resected liver tissue to gain information about the risk of persistent parasitic activity during the follow-up.

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Figure legends

Figure 1. Anti-Em18 levels before and after surgery.

Figure 2. Evolution of the serological markers by paired analysis, before and after surgery. A. Evolution of anti-Em18 levels before and after surgery. B. Evolution of PD-L1 levels before and after surgery. C. Evolution of PD-1 levels before and after surgery.

Figure 3. Microarray results and decline of anti-Em18 levels. NS=non significant.

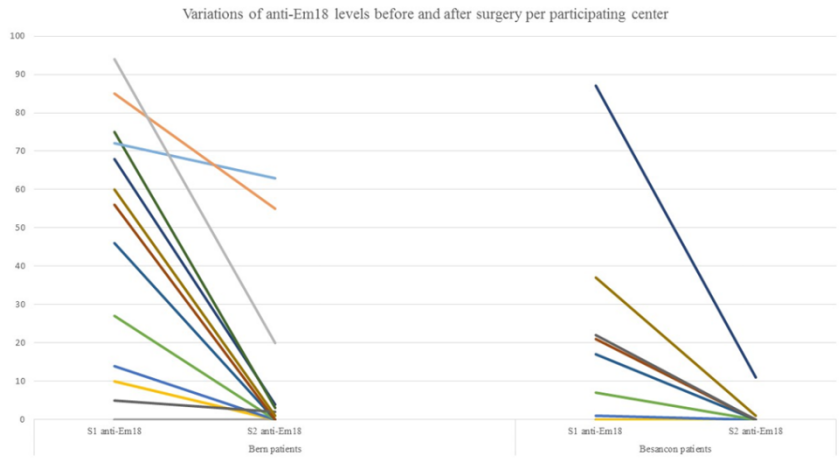
Tables

Table 1. Description of the patient's management and evolution per center of recruitement

Bern (Switzerland) n=26	
Age (median [range])	56 [22-75]
Symptomatic at diagnosis	69%
PNM scoring 100	39%
PNM scoring 200	35%
PNM scoring 300	11%
PNM scoring 400	15%
Complete resection (R0 surgery)	88%
Primary size of the lesions (cm)	4.5 [0-20]
Immunosuppression	38%
Immuno-suppressive medication before surgery	7.7%
Time between diagnosis and surgery (median [range])	2.8 months [0 day to 1.4 years]
Most frequent type of imaging	CT scans (88%)

Time between diagnosis and albendazole initiation (median [range])	19 days [0 day to 3 months]
Duration of albendazole treatment (median [range])	1.6 years [1.9 months to 3.1 years]
Follow-up time (median [range])	2.1 years [4 months to 6.2 years]
Persistent residual parasitic activity	47%
Total disappearance of residual parasitic activity	53%
Besancon (France) n=14	
Age median [range]	61 [22-73]
Symptomatic at diagnosis	43%
PNM scoring 100	65%
PNM scoring 200	14%
PNM scoring 300	0%
PNM scoring 400	21%
Complete resection (R0 surgery)	50%
Primary size of the lesions (cm)	4.75 [2.3-8.5]
Immunosuppression	7%

Immuno-suppressive medication before surgery	0%
Time between diagnosis and surgery (median [range])	4 months [0 day to 6.6 years]
Most frequent type of imaging	CT scans (50%)
Time between diagnosis and albendazole initiation (median [range])	1.5 months [0 day to 6 months]
Duration of albendazole treatment (median [range])	2.3 years [2.1 months to 7 years]
Follow-up time (median [range])	9.3 years [1.8 years to 13.4 years]
Persistent residual parasitic activity	18%
Total disappearance of residual parasitic activity	82%



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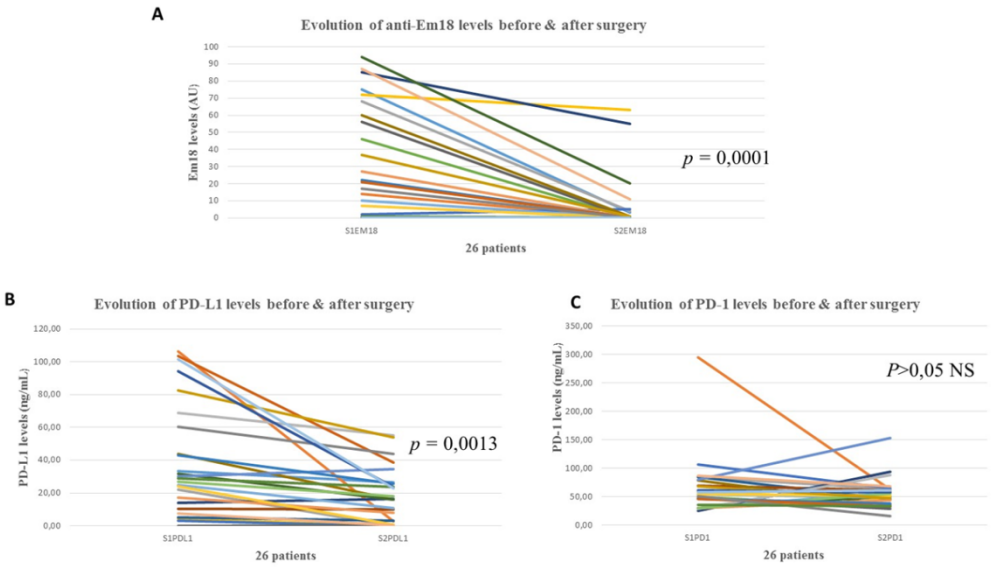

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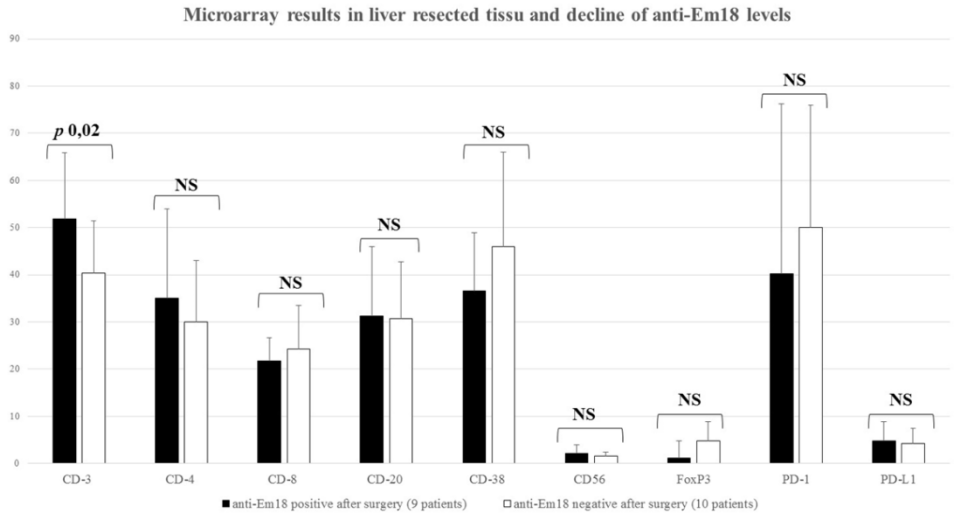

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