



Research Paper

Mesothelin-specific Immune Responses Predict Survival of Patients With Brain Metastasis



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ABSTRACT

Background: Patients with advanced malignancies, e.g. lung cancer, ovarian cancer or melanoma, frequently present with brain metastases. Clinical presentation and disease progression of cancer is in part shaped by the interaction of the immune system with malignant cells. Antigen-targeted immune responses have been implicated in the prolonged survival of patients with cancer. This includes the tumor-associated antigen (TAA) mature mesothelin, a 40 kDa cell surface-bound antigen that is overexpressed in several malignancies including lung ovarian and pancreatic cancer. We examined in an observational, prospective study the survival of patients with brain metastases in association with clinical parameters and cellular immune responses to molecularly defined TAAs or viral (control) target antigens.

Methods: Immune cells in peripheral blood obtained from thirty-six patients with brain metastases were tested for cytokine production in response to a broad panel of defined viral and TAA target antigens, including full-length mesothelin. Incubation of immune cells with antigenic targets was carried out in i) medium alone, (ii) in a cytokine cocktail of interleukin (IL)-2/IL-15/IL-21, or (iii) IL-2/IL-7. Supernatants were tested for interferon gamma (IFN- γ) production, after which univariate and multivariate analyses (Cox stepwise regression model) were performed to identify independent clinical and immunological factors associated with patient survival. Patients were followed-up for at least 500 days after surgery or until death.

Findings: Univariate analysis identified age, gender, radiotherapy and mutational load as clinical parameters affecting survival of patients with brain metastases. Cox multivariate analysis showed that radiotherapy ($P = 0.004$), age ($P = 0.029$) and IFN- γ responses to mature mesothelin, conditioned by IL-2/IL-7 ($P = 0.045$) were independent predictors of the survival of patients from surgery up to follow-up or death.

Interpretation: This is the first evidence that immune responses to mesothelin serve as a marker of increased overall survival in patients with brain metastases, regardless of the primary tumor origin. Analyses of immunological markers could potentially serve as prognostic markers in patients with brain metastases and help to select patients in need for adjunct, immunological, treatment strategies.

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1. Introduction

Approximately half of the patients with advanced lung cancer, 20% of patients with breast cancer and up to 10% of patients with melanoma present with metastatic disease in the brain (WHO. World Cancer

Report, 2014). More recent evidence suggests that between 1 and 12% of patients with high-grade ovarian cancer develop multiple brain metastases (Pakneshan et al., 2014). Several factors are known to contribute to the aggressiveness of brain metastases, among them mutations in DNA repair genes, production of angiogenic mediators, such as vascular endothelial growth factor (VEGF), and chronic inflammation which leads to aberrant activity of protein kinase pathways (Hanahan and Weinberg, 2011). Open surgery, radiosurgery (i.e. with the Gamma Knife) and/or radiation therapy of brain metastases are viable treatment options for patients with brain metastases. Patients at risk to succumb

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to their underlying disease will benefit from risk analysis that would allow to offer - in addition to radiation and/or surgery – adjunct host directed therapies, such as checkpoint inhibitors or more targeted immune therapies. A prime candidate target for clinically relevant immune responses is mesothelin, which is expressed as a 72-kDa protein and cleaved by furin to yield a 33 kDa fragment constituting megakaryocyte-potentiating factor (MPF) (Pastan and Hassan, 2014; Tchou et al., 2012). MPF is shed into the systemic circulation, and is associated attributed with megakaryocyte colony-inducing properties (Pastan and Hassan, 2014; Tchou et al., 2012). The remaining component of mesothelin ('mature mesothelin' or 'GPI') exists as a 40 kDa glycosylphosphatidylinositol (GPI)-anchored membrane-bound mature protein that is overexpressed in various solid tumors i.e. mesothelioma (Chang and Pastan, 1996), lung (Thomas et al., 2015), pancreatic (Argani et al., 2001; Hassan et al., 2005a), ovarian (Chang and Pastan, 1996; Hassan et al., 2005b) and breast cancers (Parinyanitikul et al., 2013).

In some cancers i.e. triple negative breast cancer and lung adenocarcinoma, overexpression of mesothelin is linked to poor prognosis (Thomas et al., 2015; Tozbikian et al., 2014). These clinical findings lay the ground for the development of mesothelin-based T-cell therapies for the adjunct treatment of patients with solid tumors (Newick et al., 2017). The interaction of mesothelin with mucin 16 (cancer antigen 125) forms the basis in the dissemination of cancer cells in epithelial ovarian cancer (Gubbels et al., 2006), mesothelioma (Hassan et al., 2010) as well as pancreatic cancer (Chen et al., 2013), linking the development of metastasis and the recognition of mesothelin by humoral and cellular immune responses.

However, cellular immune responses to mesothelin have not been explored as a predictive marker for survival of patients with brain metastases. We tested in this study the response of antigen-specific peripheral blood lymphocytes to a broad panel of molecularly defined target antigens and linked the immune response pattern to survival of patients with brain metastasis in order to identify patients at increased risk to succumb to their underlying disease.

2. Materials and Methods

2.1. Cohort Description

This study was approved by the Regional Ethics Review Board (Regionala etikprövningsnämnden) at Karolinska Institutet, Stockholm (ethical permit number: 2013/576-31). Thirty-six (36) patients with brain metastases arising from various primary solid tumors amenable for surgical resection were recruited between 19th January 2015 and 9th August 2016 following informed consent. The follow-up period for each patient was at least 7 months or until death. Heparinized venous blood for laboratory studies was drawn from the participating patients at surgery. The demographic variables considered were gender and age of the patients, while clinical parameters included primary tumor location, diameter of resected metastasis, presence of edema, localization of resected brain metastasis, single/multiple metastasis, number of mutation(s), Karnofsky Performance Scale (KPS) score, metastasis Recursive Partitioning Analysis (RPA) classification and radiotherapy. The full baseline description of the patient cohort is provided in Table 1.

2.2. Whole Blood Assay (WBA) and Detection of Interferon Gamma (IFN- γ) Production

Peripheral blood was diluted at a ratio of 1:1.5 with RPMI 1640 Glutamax medium (ThermoFisher Scientific, Carlsbad, CA) and supplemented with antibiotics (penicillin, 100 IU/mL and streptomycin, 100 μ g/mL) (ThermoFisher Scientific, Carlsbad, CA). Next, the diluted blood was treated either with i) human IL-7 (10 ng/mL) and IL-2 (500 IU/mL), or ii) human IL-2 (1000 IU/mL), IL-15 (10 ng/mL) and IL-21 (10 ng/mL) or left untreated (RPMI medium only). All cytokines

Table 1

Univariate analysis of demographic and clinical factors. (Kaplan-Meier estimation with log-rank test).

Factor		P value
Age		0.0687 ^a
Median	60	
Range	30–84	
Sex		0.1367 ^a
M	15	
F	21	
Primary tumor		0.8681
Lung cancer	14	
Melanoma	8	
Ovarian cancer	3	
Other	11	
Tumor diameter (cm)		0.9250
Median	4	
Range	1–6.4	
Radiology edema		0.5819
No	2	
Moderate	18	
Severe	16	
Tumor localization		0.3390
Frontal	11	
Temporal	5	
Parietal	6	
Occipital	6	
Cerebellum	8	
Single/multiple metastasis		0.1587
Single	23	
Multiple	13	
Mutation		0.1401 ^a
2	2	
1	10	
0	24	
KPS score		0.2873
>80	22	
≤80	14	
Metastasis RPA classification before surgery		0.1563
2	24	
1	12	
Extent of resection		0.5878
Complete	27	
Partial	7	
Unknown	2	
Radiotherapy		0.0053 ^a
LINAC - yes	18	
LINAC - no	18	

KPS: Karnofsky Performance Status; RPA: Recursive Partitioning Analysis.

^a Included in multivariate analysis.

were purchased from Prospec (Ness-Ziona, Israel). The cytokine-treated as well as the untreated blood samples were added to 96-well round-bottom plates pre-coated with a panel of antigens (Supplementary Table 1), followed by incubation at 37 °C with 5% CO₂ for seven days. Antigen-free medium was used as negative control while 5 μ g/mL phytohaemagglutinin (PHA, Sigma-Aldrich, St. Louis, MO), 30 ng/mL OKT3 (anti-human CD3 monoclonal antibody, BioLegend, CA) or 10 ng/mL SEA + SEB (Staphylococcal Enterotoxin A and B, Sigma-Aldrich, St. Louis, MO), respectively were used as positive controls. Measurement of IFN- γ production by immune cells after antigen exposure was performed with a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (MABTECH, Stockholm, Sweden).

2.3. Statistical Analysis

All the demographic, clinical and immunological (target – specific IFN- γ production) parameters served as potential prognostic factors, and were evaluated in relation to survival of the patients using Kaplan-Meier estimators, while P values were generated using log-rank tests. Cut-off points for continuous univariate factors were generated based on the greatest hazard ratio between two sub-groups.

Subsequent to the univariate analysis, all factors with a P value < 0.15 were considered to potentially influence patient survival and were therefore introduced into the Cox proportional hazards model for forward and backward stepwise multivariate analysis. Each immunological univariate factor (i.e. antigen stimulation, OKT3 control etc.) with P < 0.15 obtained from the three WBA culture conditions (untreated, IL-2/IL-15/IL-21 or IL-2/IL-7) were then individually combined with the demographic and clinical parameters for the stepwise multivariate analysis. All independent factors with P < 0.05 were considered as having an effect on survival of the patients, while non-significant factors were removed for the final data compilation.

2.3.1. Role of the Funding Source

The funding source did not have any role in the conception and conduct of the study, nor the interpretation of results and writing of the manuscript.

3. Results

The clinical profile of the patient cohort is provided in Table 1. Patients with metastases to the brain (n = 36) had primary tumors in the lung, ovaries (epithelial ovarian cancer), skin (melanoma) or other organs. The median age of the patients was 60 years, with a gender distribution of 15 males and 21 females. Post-operatively, eighteen out of thirty-six (18/36) patients underwent linear accelerator (LINAC) radiotherapy (i.e. fractionated radiotherapy (FRT) or WBRT). The remaining 18 patients did not receive LINAC radiotherapy but rather GKS (radio-surgery) or none of the above after open surgery. Twenty-two patients presented with a > 80 score on the Karnofsky Performance Scale (KPS), indicating their dire clinical condition, while the rest of the patients (n = 14) had a KPS score below 80. Two of the thirty-six patients had two mutations in the genome, ten patients exhibited one mutation and the remaining patients (n = 24) had none. Using the Kaplan-Meier estimator (KME) with long-rank test, each of the clinical parameters presented in Table 1 were tested for an existing relationship with survival of the patients with brain metastases

with a P value cut-off under 0.15. Of these, only four (age, gender, presence of mutations and radiotherapy) obeyed the cut-off value, and were therefore selected as the basis for further analyses (Table 1).

We also evaluated IFN- γ production in response to stimulation of peripheral blood with the mature mesothelin peptide pool, in the presence or absence of cytokine conditioning (IL-2 and IL-7 or IL-2, IL-15 and IL-21). There was no marked enhancement in the median amount of IFN- γ produced by lymphocytes in response to mesothelin stimulation regardless of cytokine conditioning of peripheral blood (Supplementary Fig. S1). The proportion of patients who were able to produce detectable levels (≥ 2 pg/mL) of mesothelin-specific IFN- γ WBA was comparable between the three groups (without cytokines = 11 patients, IL-2/IL-7 conditioning = 14 patients, IL-2/IL-15/IL-21 conditioning = 11 patients). The analysis suggested mesothelin as a univariate factor with an effect on the patient survival pattern with the IL-2/IL-15/IL-21 and IL-2/IL-7 culture conditions (Fig. 1). Other immunological univariate factors apart from mesothelin-directed immune responses, which were included in the Cox stepwise model with a cut-off of P < 0.15 are shown in Supplementary Table 2. The immunological univariate factors from the respective WBA culture conditions were then combined with the four demographic and clinical factors (age, gender, number of mutations and radiotherapy) which were determined via the univariate analyses to carry out the Cox proportional hazard model multivariate analysis. Forward and backward Cox stepwise modelling were used in order to determine whether single, independent parameters within a group of parameters can directly influence the length of patient survival (Sperduto et al., 2017). The results of the multivariate analysis confirmed that radiotherapy is an independent predictor of longer survival time (P = 0.004). Importantly, a positive correlation between detectable mesothelin-specific IFN- γ responses of lymphocytes and increased survival of patients with brain metastasis was observed exclusively in the presence of IL-2 and IL-7 conditioning of peripheral blood in the WBA (P = 0.045), along with the age of the patients as an independent demographic factor (P = 0.029) (Table 2). Of note, increased survival was only observed in association with immune reactivity to the 'mature', cell-associated part of the mesothelin protein and not

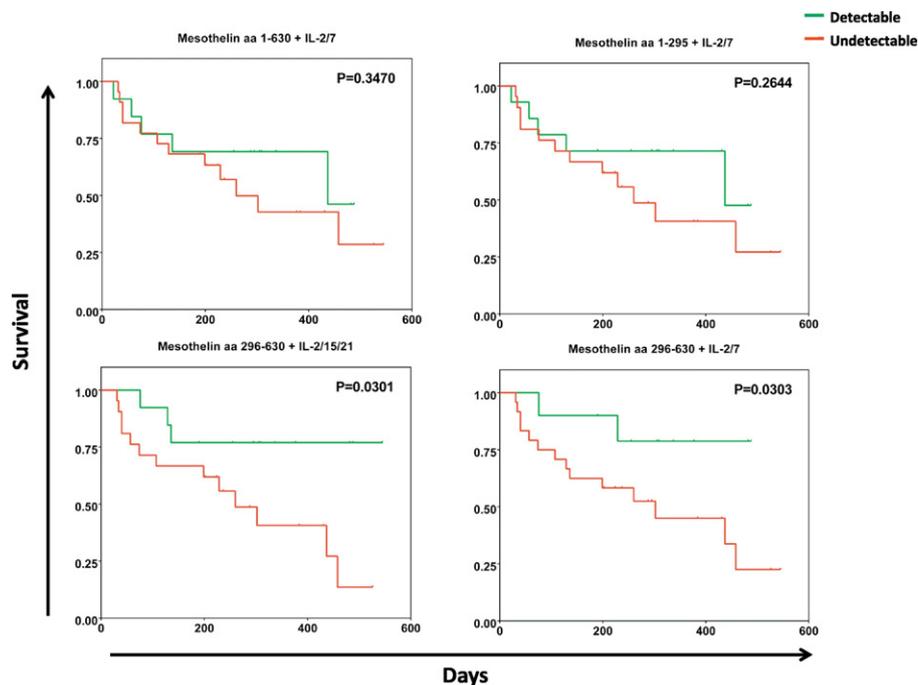


Fig. 1. Survival pattern of patients with brain metastases in relation to mesothelin-specific IFN- γ production based on univariate analysis. Kaplan-Meier survival curves show the relationship between IFN- γ responses to mature mesothelin and survival of the patients. Survival advantage associated mesothelin(GPI)-specific IFN- γ production was observed at 30 days post-surgery until the end of clinical follow-up or death, in the presence of IL-2/IL-15/IL-21 or IL-2/IL-7 conditioning of peripheral blood in the WBA. P < 0.05 was considered significant. The IFN- γ production against full-length mesothelin or MPF is shown as controls.

Table 2

Forward and backward stepwise model confirmed prognostic variables (Cox proportional hazards model).

Stepwise (COX)	HR	P value	95% CI	
Radiotherapy	0.1488	0.004	0.04075	0.54339
Age	0.2310	0.029	0.0621	0.85923
Mature mesothelin	0.2566	0.045	0.0678	0.9716

with the MPF part, nor with other, frequently expressed TAAs in solid cancers, e.g. NY-ESO-1, survivin or viral (CMV, EBV) (control) target antigens.

We also performed flow cytometric analysis to evaluate whether the tumor cells isolated from metastases expressed mature mesothelin on their surface. Four out of five tumor cell lines tested, established from resected tissue originating from patients with metastatic lung or ovarian cancer, displayed strong expression of mature mesothelin on the surface after *ex vivo* propagation, compared to the day of cell isolation and initiation of culture (Supplementary Fig. 1). This was not observed for tumor cell lines established from resected brain metastases from patients with advanced melanoma.

4. Discussion

This study is the first to establish a link between mesothelin-specific cellular immune responses and improved survival of patients with brain metastasis. The observation that cytokine conditioning of peripheral blood T cells (with IL-2/IL-15/IL-21 or IL-2/IL-7 combinations) from patients with brain metastases led to increased IFN- γ responses directed against mesothelin shows that: (i) TAA-specific immune responses can be amplified *in vitro*, (ii) these immune responses are clinically relevant and (iii) such immune responses could also be amplified for active cellular therapy for treating patients with advanced cancer.

The pronounced survival benefit provided by the use of radiotherapy to treat patients with brain metastases is well established (WHO. World Cancer Report, 2014; Hall et al., 2000). It has been previously been reported that patients with brain metastasis, originating from ovarian carcinomas or melanoma, as well as non-small cell carcinoma of the lung exhibit an increased 2-year survival rate when treated with surgical resection in combination with radiotherapy (Hall et al., 2000). Abscopal effects following radiotherapy in patients with metastatic cancer have been reported for several decades, where immune-related disease-modifying activity causes tumors outside the focal area of radiotherapy to regress, potentially by improving antigen processing and presentation as well as by activation of TAA-specific T-cell activation (Siva et al., 2015; D'Souza et al., 2016). Radiotherapy also induces systemic reduction in circulating FoxP3+ regulatory T cell (Treg) numbers, which when elevated, indicate a poor prognosis for patients with metastases to the brain (D'Souza et al., 2016; Hamilton and Sibson, 2013).

The 'conditioning effect' of the gamma chain cytokines IL-2 and IL-7 on peripheral blood T-cell homeostasis is significant. The therapeutic use of IL-2 in various human cancers has been long documented, being indispensable for T-cell proliferation directed against transformed cells (Rosenberg, 2014; Quattrocchi et al., 1999; Sakamoto et al., 2011; Takayama et al., 1991). Patients with chronic infectious disease indications, characterized by impaired and/or dysfunctional T-cell responses such as pulmonary tuberculosis and viral hepatitis, may also benefit from IL-2 therapy (Shen et al., 2015; Tomova et al., 2009) in part by reconstituting the T-cell receptor zeta chain (TCR ζ) expression, a pivotal link for T-cell activation and 'immune fitness'. IL-7 has been shown to increase expansion of circulating human CD4 and CD8 T cells, with a positive effect on T-cell receptor (TCR) repertoire diversification and reduction in Treg numbers (Sportes et al., 2008). Furthermore, recent pre-clinical studies have shown that IL-7 treatment promotes CD4 T cell poly-functionality leading to tumor containment (Ding et al., 2016). In combination with IL-12 pre-priming of antigen-experienced CD8 T

cells, IL-7 can induce cellular proliferation after IL-12 withdrawal (Johnson et al., 2016). Thus, both IL-2 and IL-7 have a direct influence on fine-tuning the repertoire of anti-cancer T-cells in the host.

Based on the findings of the present study, we propose that systemic, biologically relevant anti-mesothelin T-cell responses exist in patients with brain metastasis and that these TAA-specific immune responses can be augmented by IL-2 and IL-7, as well as by the cytokine combination of IL-2, IL-15 and IL-21. The potency to increase the quality of mesothelin – specific T-cells, reflected by antigen-specific IFN γ production is clinically relevant, since increased IFN γ has been reported to be associated with better responses to immunotherapy in a smaller study of patients with pancreatic cancer. The combination of IL-2 and IL-7 may lead to the preferential expansion of T cells with target-specific TCRs, recognizing cancer epitopes that are involved in the pathogenesis of brain metastases. This might not necessarily be reflected in the primary tumor itself i.e. lung, ovarian cancers that may exhibit a different gene expression profile, although this hypothesis requires formal testing.

Improved management of patients with brain metastases may consider incorporating mesothelin-specific cellular immune responses as a predictor of survival; patients at risk to succumb earlier to brain metastases may be offered adjunct therapies. T cells recognizing mesothelin epitopes may be developed as immunotherapeutic strategies and patients with brain metastases lacking anti-mesothelin responses may benefit from alternate treatment options. Furthermore, this study lays the foundation for discovering other biologically and clinically relevant cellular target antigens for patients with brain metastases, which may further improve patient care and provide the molecular and immunological tools for better health care decisions. The present study underlines that not only the biological profile of the malignant disease, e.g. mutational load, yet also that 'immune cell fitness' of the host impacts on overall survival.

5. Conclusion

We show that qualitative tailoring of mesothelin-specific cellular immune responses by IL-2 and IL-7 correlate with extended survival of patients with brain metastases providing a tool for a more tailored follow-up of these patients and flagging the need to individually tailor potential adjunct therapies in addition to surgery and radiotherapy. This finding also warrants the further exploration of the TCR repertoire of memory T cells in patients with brain metastases who survive and the potential use of mesothelin – directed active cellular therapy for patients with brain metastases.

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Conflicts of Interests

The authors declare no conflicts of interests.

Author Contributions

L.Z., I.-H-P., E.D. and M.M., conceived the study. J.B.Jr., G.S., I.-H-P., and E.D. arranged for and provided clinical samples. L.Z., X.L., E.Sa., E.Sc., Q.M., A.v.L. and T.P. performed the experiments and analyzed the data. D.V. provided expert assistance related to statistics. L.Z., M.R., M.M., J.B.Jr., E.D., and I.-H-P. structured and wrote the paper.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2017.08.024>.

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