Serum concentration of integrin-linked kinase in malignant pleural mesothelioma and after asbestos exposure†

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Abstract

OBJECTIVES: Integrin-linked kinase (ILK) is an intracellular protein implicated in chronic inflammation and neoplastic transformation. In a recently accomplished pilot study, we showed that ILK can be detected in the serum of patients with benign and malignant chest diseases, including malignant pleural mesothelioma (MPM). Interestingly, average serum ILK concentrations were 10 times higher in MPM patients when compared with the rest of the study population, and a diagnostic test solely based on serum ILK concentration could discriminate between MPM and non-PMMP with considerable accuracy. This study aimed to investigate whether serum ILK concentration could also be used to discriminate between MPM and asbestos exposure only.

METHODS: Using a self-developed sandwich enzyme-linked immunosorbent assay, we measured serum ILK concentrations in 101 MPM patients, and 96 asbestos-exposed, but healthy insulation workers. Seventy-three MPM patients had an epitheloid subtype (72.3%), and 42 had a Stage I or II disease (41.6%).

RESULTS: When compared with asbestos-exposed individuals, MPM patients of all clinical stages had significantly higher (mean ± standard deviation, median) serum ILK concentrations (10.7 ± 13.6, median 7 ng/ml vs 3.1 ± 4.6, median 1.4 ng/ml; P < 0.001). Among MPM patients, the serum ILK concentration was significantly higher at advanced disease stages III + IV than at early stages I + II (13.7 ± 15.9, median 8.5 ng/ml vs 6.7 ± 7.8, median 3.5 ng/ml; P = 0.02). Using serum ILK to discriminate between MPM patients and asbestos-exposed individuals yielded an area under the curve of 0.69 (95% confidence interval 0.63–0.76). The corresponding sensitivity and specificity for a cut-off of 4.49 ng/ml ILK are 61.4 and 80.2%, respectively.

CONCLUSIONS: These data show significant differences between MPM patients and asbestos-exposed but healthy individuals concerning their serum ILK concentration. Furthermore, since ILK levels are increased in advanced MPM stages in comparison with early MPM stages, we suggest evaluating its potential use as a marker of disease progression in MPM.

Keywords: Malignant pleural mesothelioma • Integrin-linked kinase • Asbestos exposure • Differential diagnosis • ELISA

INTRODUCTION

Despite intensive multidisciplinary therapy efforts [1–3], the outcome of malignant pleural mesothelioma (MPM), a tumour originating from the pleura and mostly associated with asbestos exposure, remains extremely poor [4], because a majority of patients present with advanced disease. Thus, early detection by reliable biomarkers may be a valuable strategy to improve the outcome in MPM patients. Since chronic inflammation of the pleura induced by asbestos exposure is the basic pathogenetic principle of MPM [5], a suitable biomarker for early MPM detection would potentially be a protein involved in both inflammatory and neoplastic processes.

The cytoplasmatic integrin-linked kinase (ILK), a serine/threonine-kinase that links β1-integrins to the cytoskeleton [6], may satisfy these criteria. It not only participates in the inflammatory signalling cascade [7], but can also induce an epithelial-mesenchymal transition and a tumourigenic phenotype in epithelial cells, if overexpressed [8]. Clinically, ILK is involved in many inflammatory and neoplastic diseases [9, 10].
Regarding ILK and MPM, we showed that, when compared with healthy pleural tissue, ILK is strongly expressed in MPM specimens [11, 12]. Subsequent prognostic modelling, however, did not show associations between ILK and time-to-death, [12]. Interestingly, we also detected ILK in the serum of patients with MPM and with other benign or malignant chest diseases [13]; average serum ILK concentrations were 10 times higher in MPM patients when compared with the rest of the study population. Furthermore, a diagnostic test solely based on serum ILK concentration could discriminate between MPM and non-MPM diseases with considerable accuracy. However, in order to evaluate the real potential of serum ILK for early MPM detection, a comparison of serum ILK concentration in established MPM and in healthy subjects at risk for MPM development is necessary.

Therefore, this study aimed to investigate whether serum ILK concentration can be used to discriminate between established MPM and asbestos exposure only.

**MATERIALS AND METHODS**

The study was approved by the institutional review boards of the Otto Wagner Hospital, Vienna, Austria, and of the New York University, New York City, USA. All participating probands had signed written informed consent before donating a vial of venous blood for storage in the thoracic surgical tissue bank at NYU Langone Medical Center in USA and for subsequent prognostic marker isolation. From this thoracic surgical tissue bank, frozen serum samples of 101 patients with MPM and of 96 asbestos-exposed, but healthy insulation, workers were retrieved (Table 1). The blood of MPM patients was drawn at the time of surgery, prior to the incision. The blood of asbestos-exposed but healthy individuals was drawn after a chest computed tomography (CT), excluding the presence of MPM. The following clinical parameters were recorded: age, gender, combined stage of MPM.

The frozen serum samples were transferred to the Cardiac Surgery Research Laboratories, Medical University of Vienna, Vienna, Austria, for subsequent serum ILK analysis. The laboratory personnel responsible for the serum ILK analysis were blinded to the identity and to the clinical conditions of the participating patients. Serum ILK concentrations were measured using our self-developed sandwich enzyme-linked immunosorbent assay (ELISA) [13]. Briefly, standard 96-wells microtiter plates (Nunc®, Immuno Plates Maxi Sorp C96; art. nr. 430341) were coated with a solution of a capture antibody (Sigma-Aldrich®, art. nr. I 783 mouse/anti-ILK/monoclonal) and incubated overnight. After incubation, native serum specimens and an ILK standard curve were incubated for 1 h followed by the subsequent addition of a polyclonal detecting antibody (Sigma-Aldrich®, art. nr. I 1907, rabbit/anti-ILK) and a polyclonal conjugated antibody (Dako®, IgG-HRP, goat/anti-rabbit). Absorbance measurement was performed on a multilabel plate reader (Victor3 model nr. 1420, PerkinElmer®) at a wavelength of 450 nm, followed by the calculation of serum ILK concentration by a previously established standard curve model. ILK ELISA was performed in two separate runs for every sample, and—additionally—in two wells per run.

### Statistical analysis

All calculations were performed using SPSS 19.0. Metrical data like ILK concentration or age are described using mean ± standard deviation when normal distributed or mean ± standard deviation, median and maximum/minimum when skewed. Nominal data are presented using absolute numbers and percentages. To compare the three groups, Welch-corrected analyses of variance (ANOVA) were assessed. For ILK, two a priori contrasts were defined. The first comparing asbestos-exposed individuals with mesothelioma patients, the second comparing mesothelioma patients Stages I and II with mesothelioma patients Stages III and IV. Post hoc tests according to Games Howell were used to do post hoc comparisons for age. A Fisher-Halton-Freeman test was performed to compare the gender distribution between the three groups. However, as all healthy asbestos-exposed individuals were of male gender, we could not model an interaction between group and gender. Therefore, we additionally performed three analyses of covariance (ANCOVA) using age as a covariate to assess the impact of age and gender. For the first ANCOVA, only main effects are modelled for age, group and gender (which is equal to a multiple regression analysis), in the second ANCOVA, only men and in the third ANCOVA, only patients with mesothelioma were used. Furthermore, a receiver operating characteristic (ROC) analysis has been performed. A P-value equal to or below 0.05 was considered to indicate significant results.

<table>
<thead>
<tr>
<th>Demographic characteristics of study groups</th>
<th>Asbestos exposure (n = 96)</th>
<th>MPM Stages I and II (n = 42)</th>
<th>MPM Stages III and IV (n = 59)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years; mean ± standard deviation)</td>
<td>57.8 ± 9.6</td>
<td>66.1 ± 12.5</td>
<td>62.8 ± 9.2</td>
<td>0.001*</td>
</tr>
<tr>
<td>Gender (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>100</td>
<td>73.2</td>
<td>67.2</td>
<td>&lt;0.001b</td>
</tr>
<tr>
<td>Female</td>
<td>26.8</td>
<td>26.8</td>
<td>32.8</td>
<td></td>
</tr>
<tr>
<td>Histological subtype of MPM (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epitheloid</td>
<td>73.8</td>
<td>71.2</td>
<td>72.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Other</td>
<td>26.2</td>
<td>26.8</td>
<td>28.8</td>
<td></td>
</tr>
</tbody>
</table>

*The reported P-value refers to the difference between the ages of patients with asbestos exposure and all patients with MPM; there were no significant differences of age between early (I + II) and advanced (III + IV) MPM stages (P > 0.31).

bThe reported P-value refers to the difference in gender distribution between individuals with asbestos exposure and all patients with MPM. MPM: malignant pleural mesothelioma.
RESULTS

Patients with asbestos exposure were significantly younger than those with both MPM stage groups (P = 0.001 and 0.006, respectively). However, there was no significant difference in age within MPM stage groups (P = 0.31; Table 1). Patients with asbestos exposure were exclusively of male gender, while MPM patients had a male-to-female ratio of about 2.5:1 (P < 0.001). Seventy-three MPM patients had an epitheloid subtype (72.3%), and 42 had a Stages I or II disease (41.6%).

MPM patients of all clinical stages had a significantly higher serum ILK concentration (10.7 ± 13.6, median 7 ng/ml) when compared with asbestos-exposed but healthy individuals (3.1 ± 4.6, median 1.4 ng/ml; P < 0.001; Table 2). Among the group of MPM patients, serum ILK concentrations were significantly higher at advanced clinical stages (III + IV) when compared with early clinical stages (I + II) (13.7 ± 15.9, median 8.5 ng/ml vs. 6.7 ± 7.8, median 3.5 ng/ml; P = 0.02; Fig. 1). As for the possible influence of age and gender, in none of the ANCOVAs age significantly influenced serum ILK concentration (P = 0.901, 0.436 and 0.706). Furthermore, there was no statistical impact of gender on serum ILK concentration (P = 0.245). Furthermore, no significant differences of serum ILK concentrations were found with respect to the histological subtype (P = 0.7).

A serum ILK concentration cut-off at 4.49 ng/ml distinguished between asbestos exposure and MPM (all stages) with a sensitivity of 61.4% (62 of 101) and a specificity of 80.2% (77 of 96) area under the curve [(AUC) = 0.69; 95% confidence interval 0.63–0.76; Fig. 2].

DISCUSSION

In this study, we could show that serum ILK concentration can be used to discriminate between established MPM and asbestos exposure only, the latter having a lower serum ILK concentration than the former. In addition, we found that serum ILK concentration in advanced MPM stages is increased in comparison with that in early MPM stages.

These results align very well with the literature and our previous investigations. In 2008, we described high immunohistochemical expressions of ILK in 90% of MPM tissue samples, regardless of the histological subtype, whereas healthy pleural and lung tissue did not express ILK at all [11, 12]. Another interesting point of our results becomes evident upon comparison with our previous findings: the observed median serum ILK concentration was only 3.1 ± 4.6 and 1.4 ng/ml in healthy control individuals.

Table 2: Overview of serum ILK concentrations

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asbestos exposure</td>
<td>3.1</td>
<td>4.6</td>
<td>1.4</td>
<td>0</td>
<td>20.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MPM (all stages)</td>
<td>10.7</td>
<td>13.6</td>
<td>7</td>
<td>0</td>
<td>77.8</td>
<td></td>
</tr>
<tr>
<td>Histological subtype of MPM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epitheloid</td>
<td>10.6</td>
<td>14.0</td>
<td>5.9</td>
<td>0</td>
<td>77.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Other</td>
<td>10.7</td>
<td>12.3</td>
<td>9</td>
<td>0</td>
<td>49.7</td>
<td></td>
</tr>
</tbody>
</table>

MPM: malignant pleural mesothelioma; SD: standard deviation.
concentration in asbestos-exposed but otherwise healthy individuals (1.4 ng/ml) is almost double the reported value of 0.78 ng/ml in patients with benign chest diseases (including empyema) and of 0.66 ng/ml in patients with non-MPM malignant chest diseases [13]. These findings suggest a specific association between asbestos exposure and ILK.

The hypothesis of an asbestos-ILK association gets further credit from in vitro evidence. Upregulated ILK has been found in rat tissues and cells after asbestos treatment [14]. Furthermore, in the pleural mesothelial cell line 4/4 RM-4 asbestos exposure induced activation of the extracellular signal-regulated kinases (ERK1/ERK2) and of protein kinase B (AKT)—a known downstream target of ILK—in a complete β1 integrin-dependent manner [15], thus supposedly via ILK. Consecutively, the activation of the ERK1/ERK2 kinases leads to an increased activator protein-1 activity and ultimately to mesothelial cell mitosis [16]. Another variant of how asbestos induces MPM is related to TNF-α. Pleural macrophages release TNF-α upon phagocytosis of asbestos fibres [17]. After binding to its receptor, TNF-α activates the NF-kB pathway in mesothelial cells leading to increased cell division [18]. In cultured myoblasts, TNF-α-related signalling was reduced by ILK inhibition, while stimulation of myoblasts by TNF-α directly increased ILK activity [19].

Synoptically, ILK seems to be an integral part of an important signalling pathway involved in MPM pathogenesis as a response to asbestos exposure. Whether ILK might be a valid pharmacological target for MPM prevention and/or treatment must be elucidated in further studies.

A limitation of serum ILK determination for early MPM detection is the modest sensitivity of discrimination. One reason for this low sensitivity could be the considerable variance of serum ILK concentration values. Since ILK is ubiquitously expressed [10], and since we know neither the normal serum ILK concentration nor the other physiological or disease processes that might contribute—at least as background—to serum ILK release, sequential serum ILK determination might be more meaningful in terms of both the diagnosis and prognosis of MPM. On the other hand, even upon histopathological examination, it is sometimes very difficult to discriminate between chronic pleuritis and established MPM [20]. Since ILK is so closely related to both inflammatory and neoplastic processes, it is conceivable that serum ILK concentrations of asbestos exposure only and of early stage MPM overlap.

It is important to point out, however, that the true performance of serum ILK determination for early MPM detection can only be appreciated upon a calculation of the positive predictive value (PPV) and the negative predictive value (NPV) [21], and this can only be achieved when the true prevalence of MPM within the investigated population is known. A working example: in our cohort, the prevalence of MPM is ~50% (which is of course much higher than in the true population at risk), thus resulting in a PPV of 76% and a NPV of 66%. Based on the calculation on a better estimate of MPM prevalence in a risk population (e. g. ~5% in a cohort of former miners [22]), the resulting PPV would decrease to 14%, while the NPV would increase to 96%.

The true MPM prevalence is controversial, but most likely considerably lower (www.orpha.net), thus resulting in other values for PPV and NPV again. In summary, MPM being a rare disease renders efforts of early MPM detection extremely difficult.

The observation that serum ILK levels were increased in advanced when compared with early MPM stages suggests that there could be a direct correlation of serum ILK concentration with the tumour burden. It must be pointed out, however, that clinical stage is only a very rough approximation of the tumour burden, which is best determined by three-dimensional CT (3D-CT) determination of tumour volume [23]. It is plausible to assume that a strong correlation of serum ILK with tumour volume as determined by 3D-CT could be found, and this should thus be verified in future studies.

In conclusion, our data show significant differences between MPM patients and asbestos-exposed but healthy individuals in terms of their serum ILK concentration. Since ILK levels are increased in advanced MPM stages in comparison with early MPM stages, we suggest evaluating its potential use as a marker of disease progression in MPM.

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Conflict of interest: none declared.

REFERENCES

APPENDIX: CONFERENCE DISCUSSION

Dr. D. Sugarbaker (Boston, MA, USA): The desire to obtain a serum marker of either early detection or disease recurrence in mesothelioma has been an elusive goal. Those in the field have followed the course of mesothelin and osteopontin as they arose initially as markers to be used, for instance, in smokers, and we know exactly which population we are interested in screening.

In previous work, your lab established that integrin-linked kinase (ILK) was present in high concentration in mesothelioma tissue samples as compared with normal pleura. In this paper, you examined ILK levels in serum of mesothelioma patients and in asbestos-exposed insulation workers using an ELISA assay. Although the mean serum ILK level was found to be more than twice higher in MMP patients than in asbestos-related controls, the distributions overlapped considerably and included undetectable ILK levels in both groups. This limits the assay’s sensitivity somewhat for detecting MMP patients among individuals at overall risk for the disease. The authors appropriately acknowledge this limitation with respect to the test’s potential role in early disease detection.

A secondary finding is that ILK levels in patients with clinical stages III and IV disease are higher than those in patients with clinical stage I and II. MMP, and this is interpreted to indicate that ILK may reflect tumour burden and therefore may be a useful marker of disease recurrence. Although this hypothesis may merit investigation, the stated rationale is somewhat speculative. Staging of malignant pleural mesothelioma is based on tumour involvement of specific structures rather than on tumour burden per se. However, it is becoming increasingly clear that tumour volume is a primary driver of prognosis, suggesting that your approach may yield significant fruit.

It would be useful to know whether serum ILK levels are reduced following surgical resection. An alternative, paroxysmal interpretation of all this data should be considered in the discussion. Serum ILK levels may be indicative of the degree of chronic inflammation that is variably present in asbestos-exposed individuals both with and without malignant pleural mesothelioma. MMP is more often a disease of the retired than of current asbestos workers and, in fact, the patient cohort of the current study is significantly older, as you pointed out in your presentation, than the insulation workers as a group. Within the given long attention span of the observers, the observed differences in ILK levels could reflect more prevalent and/or longer duration of chronic inflammation amongst the older population that you studied with MMP. I have several questions for you.

You say in your manuscript that there was a differentiation in some of the serum levels between mixed, sarcomatous and epithelial tumours. Could you clarify this statement by telling us what the differences were between the various cell types of malignant pleural mesothelioma in terms of the levels of ILK? Number two, this particular pathway, as you mentioned, has been the target, if you will, of other therapeutic interventions, and you alluded to that.

Do you see any immediate application utilizing this pathway as a potential therapeutic intervention for malignant pleural mesothelioma? You have a very detailed review and data on individual patients and their particular levels of ILK. The third question is, how do you correlate other tumour characteristics, such as the degree of pleural fluid present at presentation, with levels of ILK? This would be important, particularly since certain patients, for instance, with large pleural effusions, do not show much inflammatory clinical signs and therefore the levels may be expected to be lower.

And finally, an obvious and important collaborative study to further establish the hypothesis you have put forth would be to look at levels of gene expression for ILK in both tumour and normal, and I wonder if you have any plans to do that?

Dr. Watzka: On the question of ILK, serum ILK has been investigated in histological subtypes, and, no, we didn’t find any difference in serum ILK between histological subtypes of mesothelioma. They are not correlated to serum ILK concentrations; at least statistically we couldn’t find any detectable difference.

As for ILK and therapy, I’m glad you asked that. There are indeed therapeutic approaches underway. For ILK, small molecular inhibitors are available. Those are not blocking antibodies, against mesothelin for example. There is no therapy directed against osteopontin. But there are small molecular ILK inhibitors available. There are even phase 1, phase 2 trials of ILK inhibitors, for example, for multiple glioblastoma or other tumours (I believe it is renal cell cancer). However, the industry has some compound available. Alternatively, you could use small interfering RNA or even oligonucleotides. You can inhibit ILK selectively, and I believe personally - and this is one target of our future investigations - that we could knock it out and hopefully have some therapeutic effect on it.

As for your third question, thank you; this is a very good suggestion, looking at the fluid collection. We have not done it now, but we will certainly do it and get it out. And ILK and gene expression, there are some data, such as in vitro data, that the correlation between the expression and the final protein is pretty high. We could do it, but we didn’t do it now in this special setting.

Dr. G. Varela (Salamanca, Spain): I was thinking about the clinical applicability of your test for the diagnosis of malignant pleural mesothelioma. The sensitivity of the test is around 60%, which is not very high, but it is okay. In your series you have selected 101 MMP patients and around only 90 with inflammation, asbestos exposure. Thus the prevalence in the series is very high, because you have selected these patients. I am wondering whether in the clinical situation where the prevalence is very low, and not comparable to 60%, the positive predictive value of your test is possibly much decreased; it could be around 50%. I wonder if you think this is a reliable diagnostic test in a clinical situation?

Dr. Watzka: Certainly the sensitivity is not very high. Personally if I look at this data I interpret, this was kind of a cross-sectional analysis. We must not forget that ILK is a ubiquitous protein; it is present in all cells. We can’t exclude that there is some background production of ILK which interferes. That means for me, probably more meaningful than looking at two cohorts and comparing absolute values, following individual dynamics. So if you have patients and follow their ILK levels at certain predefined time periods, of course this has to be tested in another prospective situation, and changes in the dynamic increase in ILK correlated with the disease evolution; I think this is the one possibility of applicability regarding diagnostic potential.

As for disease monitoring, which is a second application, I think this is our next step. We will detect ILK at diagnosis, after chemotherapy, after...
surgery and so on in the follow-up, and then we will truly have an idea how the dynamic will change according to the disease process.

Dr I. Opitz (Zurich, Switzerland): I have a question. Did you assess the clinical status of these asbestos workers in more detail with CT scan, for example, whether they had other chronic inflammatory processes such as pleural plaques or asbestosis?

Dr Watzka: We did, of course, and those which were labelled as asbestos-exposed underwent a screening procedure with chest CT, and with normal blood work, but they had no evidence of anything. So they were just healthy, normal. The only difference was that they were asbestos-exposed according to their occupational situation.