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ORIGINAL ARTICLE

Assessment of the circulating klotho protein in lung cancer patients

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Abstract

The anti-aging factor, klotho has been identified as a tumor suppressor in various human cancers, including lung cancer. In vitro studies provided evidence that klotho expression influences the characteristics of lung cancer cells, however, in vivo results are lacking. The aim of our study was to evaluate whether circulating klotho protein might serve as a potential biomarker of lung cancer. Blood samples were taken from 45 newly diagnosed lung cancer patients (31 NSCLC, 14 SCLC) and 43 control subjects. Plasma klotho concentration was measured using ELISA. No difference in plasma klotho values was detected between patients and control subjects (366.3 (257.9–486.8) vs. 383.5 (304.6–489.7) pg/ml respectively (median (IQR)); $p > 0.05$). Plasma klotho levels in patients with distant metastasis did not differ from less advanced stage disease (354.2 (306.9–433.3 vs. 328.5 (242.5–419.7) pg/ml, $p > 0.05$). In contrast, analyzed with one-way ANOVA, significant difference ($p = 0.04$) was found between the examined histological types of lung cancer: adenocarcinoma (353 (329.4–438.5) pg/ml), squamous cell carcinoma (308 (209.6–348.1) pg/ml) and small cell lung cancer (388.8 (289.9–495.4) pg/ml). However, Tukey's post hoc test did not reveal significant difference between any pairs of histological groups. There was no difference between any histological subtype and health either. Our results suggest that circulating klotho protein cannot be considered as a biomarker for lung cancer. Further studies are warranted in order to examine the relationship between klotho expression in lung tissue and circulating levels of the protein, and to explore its mechanism of action in lung cancer.

Keywords Lung cancer · Biomarkers · Tumor suppressor · Premature aging · Insuline-like growth factor 1

Introduction

The anti-aging gene klotho is responsible for systemic and organ-specific aging, and has recently been identified as a tumor suppressor in various human cancers, such as cervical [1], ovarial [2] and breast [3] cancer. There is also growing evidence about its possible role in lung cancer [4, 5].

The klotho gene has two alternative transcript forms: the transmembrane and the secreted protein. The transmembrane form may undergo enzymatic cleavage releasing the extracellular part into the blood stream. This, together with the secreted protein are called soluble klotho [6] which inhibits the IGF-1/insulin pathway [7] and the Wnt/ßcatenin signaling pathway [8], both of which playing a role in lung carcinogenesis [9, 10]. As the inhibition of these pathways is of therapeutic interest [11, 12], a thorough understanding of the role of klotho in lung cancer would be important.

Klotho gene expression is reduced in lung cancer cell lines as well as in resected lung cancer tissues compared to the normal lung [13] and has been associated with survival in various lung cancers [14, 15].

Klotho influences proliferation, apoptosis [16], motility [13] and drug resistance of lung cancer cells [17] in vitro. Overexpression reduces malignant cell growth [13], while gene silencing enhances tumor development [16]. Decreased

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klotho expression has been found in drug-resistant lung cancer cell lines [17, 18], and modification of klotho expression altered proliferation of cisplatin-resistant cells [18], suggesting that klotho might have the potential to enhance sensitivity of lung cancer cells to chemotherapy.

Despite the above promising results, in vivo studies examining the role of klotho in lung cancer are still lacking. As soluble klotho can be measured reliably from peripheral blood, the protein holds the promise to serve as a biomarker. In contrast to gene expression studies about klotho, there is little information available about circulating protein in other cancers [19]. In patients with renal cell cancer, serum klotho levels were significantly lower compared to healthy subjects, and a further decrease was observed in metastatic patients [20]. Moreover, a negative correlation was found between klotho and the tumor size, grade and stage, while tissue and circulating klotho levels correlated significantly and were associated with survival [20].

Based on the above findings, we hypothesized that similarly to the reduced mRNA levels, plasma klotho concentration might also be lower in lung cancer patients and that this might be associated with the histological type and stage of the disease. We speculated that circulating klotho might provide information on proliferative and metastasis forming potential of the tumor, on survival, and that it might also forecast platinum sensitivity, thus providing additional information for therapeutic choices.

As a first step, the current study aimed to examine whether plasma klotho levels are different in lung cancer; and if they may serve as a prognostic biomarker.

Methods

Forty-five newly diagnosed lung cancer patients participated in the study. Histological diagnosis included small cell lung cancer in 14 cases and non-small cell lung cancer in 31 cases (16 adenocarcinoma, 15 squamous cell carcinoma). Stage of the cancer has been noted. Patients suffering from chronic kidney disease, additional tumors or dying before histological classification were excluded from the study. Subjects were recruited regardless of clinical parameters which have previously been shown not to alter plasma klotho values (lung function and smoking history) [21–23]. The control group comprised of 43 subjects with negative chest imaging, and were free from any recognized tumorous diseases. Subjects' characteristics are shown in Table 1. Venous blood samples were taken into EDTA tubes from all subjects. Blood samples were centrifuged immediately after collection (1500 rpm, 10 min, 4 °C) and plasma was stored at −80 °C until further analysis. The concentration of plasma soluble klotho was measured using a commercially available ELISA kit (Human soluble α-klotho assay kit, IBL, Japan).

The study was approved by an independent ethics committee, and written informed consent was obtained from all participants.

Statistical analysis

Statistical analysis and visualization of data was performed using the software packages Statistica and Graph Pad Prism (version 4.00 for Windows, CA, USA). Subjects' characteristics were compared with Student's t-test and Chi-square test. Distribution of klotho values was tested with Kolmogorov-Smirnov test. As data showed normal distribution, any difference in klotho between two groups (healthy vs. lung cancer and metastatic vs. non-metastatic) was tested with Student's t-test. One-way ANOVA on ranks was used to compare the three histological types, followed by the Tukey's post-hoc test. Minimal required sample size for comparing lung cancer and healthy subjects was calculated using the mean (562 pg/ml) and standard deviation (146 pg/ml) of serum klotho measured in healthy adults [24] and the effect size was based on the double of the approximate interassay variability (19%) of the ELISA kit at the estimated concentration. The study was not powered to subgroup analysis of lung cancer patients.

Results

There was no difference between plasma klotho values of lung cancer patients and control subjects (366.3 (257.9–486.8) vs. 383.5 (304.6–489.7) pg/ml, respectively (median (IQR)); $p > 0.05$) (Fig. 1). We noted an outlier among the healthy subjects whose characteristics or medical history could not explain his high levels. Therefore, we decided not to exclude this value from analyses. However, exclusion did not change the differences significantly.

Analyzed with one-way ANOVA, significant difference ($p = 0.04$) was found between the three examined histological types of lung cancer: adenocarcinoma (353 (329.4–438.5) pg/ml), squamous cell carcinoma (308 (209.6–348.1) pg/ml) and small cell lung cancer (388.8 (289.9–495.4) pg/ml) (Fig. 2). However, Tukey's post hoc test did not reveal significant difference between any pairs of histological groups. Neither was there difference between any histological subtype and health, nor between SCLC and NSCLC.

Regarding disease severity, plasma klotho concentration of stage IV patients (354.2 (306.9–433.3 pg/ml) did not differ significantly from patients without a recognized distant metastasis (stage I-III) (328.5 (242.5–419.7) pg/ml) (Fig. 3).

No difference was detected between lung cancer patients suffering from COPD and the ones without airway obstruction, nor between current smokers and ex-smokers.

Table 1 Subjects' characteristics

	Patient group	Control group	p
n (m/f)	45 (23/22)	43 (15/28)	n.s.
Age (years, mean ± SD)	65.1 ± 7.9	62.3 ± 6.8	n.s.
COPD (+/-)	27/18	17/26	n.s.
FEV1 (% pred.)	62.4 ± 19.8	73.9 ± 31.6	n.s.
Smoking current / non-smoker / no data	24/13/8	14/28/1	0.005*
Histological type (stage I/II/III/IV)		n.a.	n.a.
SCLC	14 (0/0/7/7)		
ACA	16 (1/0/6/9)		
SCC	15 (0/3/7/5)		

FEV1 forced expiratory volume in 1 s, *SCLC* small cell lung cancer, *ACA* adenocarcinoma, *SCC* squamous cell carcinoma; *n.s.* non-significant; *n.a.* not applicable; *significant at $p < 0.05$

Discussion

Due to the prevalence and poor prognosis of the disease, identification of predictive and prognostic biomarkers in lung cancer are essential. Such currently used biomarkers (i.e. EGFR, ALK, PD1/PDL1, ERCC, etc.) can be investigated reliably only in histological specimens, however histological sampling cannot be performed in many patients with lung cancer. In addition, these biomarkers need to be investigated by sophisticated methods (immunochemistry, PCR) which require special skills and the results often delay due to the limited access to these facilities. Therefore, finding reliably measurable circulating biomarkers is extremely warranted.

Plasma klotho protein concentration could be regarded as a potential biomarker, as it inhibits signaling pathways contributing to carcinogenesis. In line with that, dysregulation of klotho gene was described in different cancer types (i.e. epigenetic modulations have been found in breast cancer and hepatocellular cancer [25, 26]), but it has been also shown to act as a tumor suppressor in other cancers [1]. Gene expression studies predict its potential role in lung cancer as well: klotho expression is reduced in lung cancer tissue samples and cell lines compared to healthy lungs [13].

Although the role and function of klotho protein is only partially understood, it was shown to inhibit the IGF-1 signaling pathway, most probably via blocking the autophosphorylation of IGF-1 receptor [3, 7, 27]. This mechanism of action is suggested to be a major component of both the aging inhibitory effect of klotho [7] and of the regulation of cancer cell proliferation and survival [3].

In acromegaly, growth hormone producing pituitary adenoma characterized by excessively high IGF-1 levels, patients have a nearly 10-fold plasma klotho increase [28–30] and klotho concentration significantly correlates with IGF-1 level. After surgical tumor removal, both IGF-1 and klotho levels decrease to normal range [28–30], but in case of recurrent symptoms during follow-up, both protein levels increase [30]. These findings make klotho protein a very promising candidate biomarker of the disease. IGF-1 signaling pathway plays a role in carcinogenesis as well, also in the development of lung cancer. Elevated plasma IGF-1 level was associated with an increased risk of developing lung cancer [31, 32] and patients suffering from lung cancer have been shown to have higher circulating IGF1 levels compared to healthy subjects [33], however, findings are

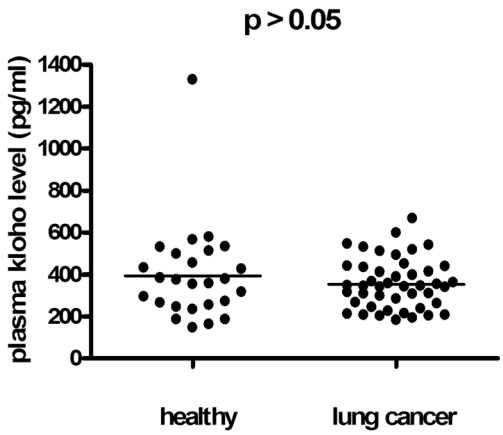

Fig. 1 Plasma klotho concentration in lung cancer patients and control subjects

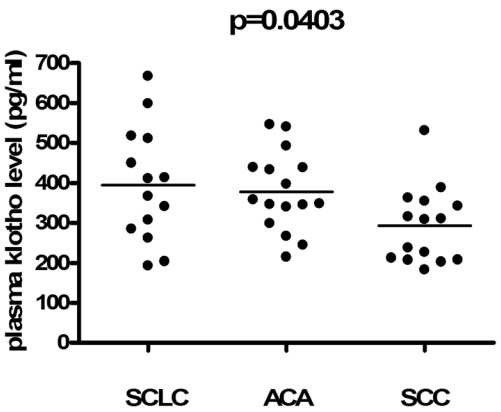

Fig. 2 Plasma klotho levels in different histological types of lung cancer

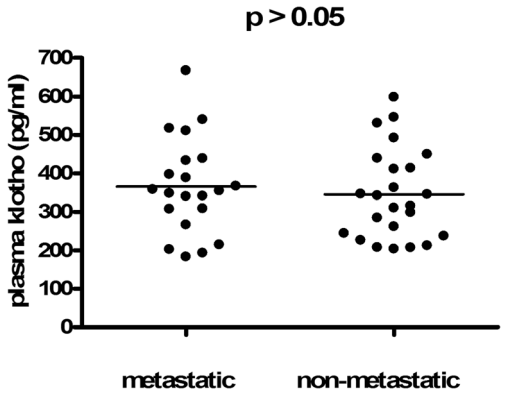

Fig. 3 Plasma klotho concentration in metastatic and non-metastatic patients

controversial in this regard [34]. Also, in lung cancer tissue, elevated IGF1 levels have been detected [35].

The above results suggested that there might be a difference between circulating klotho concentration of healthy and lung cancer subjects. Based on the reduced gene expression in lung cancer, we could expect decreased in vivo plasma klotho concentration, however elevated local and systemic IGF1 levels in lung cancer would suggest increased klotho concentration. Thirdly, klotho facilitates ischemia-induced angiogenesis [36] and klotho deficiency results in impaired angiogenesis [37] . As the protein is suggested to be protective, we could hypothesize decreased klotho level in lung cancer. Due to the various conflicting aspects, it is hard to estimate whether there might be an elevation or decrease of klotho levels, but surprisingly, we did not find any difference between healthy and lung cancer subjects. It is not clear whether this phenomenon is the result of a balance between the previous mechanisms; if the in vivo microenvironment keeps a stable concentration; whether currently unknown compensatory mechanisms exist or if circulating klotho concentration weakly represents local changes in expression. All in all, circulating klotho does not seem to be a suitable candidate biomarker for screening or early diagnosis of lung cancer.

Lung cancer patients and healthy controls tended to differ in their lung function and significantly differed in their smoking habits, which might have influenced our results. However, these parameters have previously been shown not to alter klotho levels [21–23]. Circulating klotho concentration is not influenced by current smoking [22, 23], nor correlates with the amount of smoking expressed in pack-years [22]. The presence of COPD does not influence serum klotho levels either [23], nor correlates klotho with FEV1% [22, 23]. In line with this, we did not find any difference between current smokers and ex-smokers, nor between lung cancer patients also suffering from COPD and the ones without airway obstruction.

We also expected altered klotho levels depending on the histological type of cancer. Usuda et al. found tissue klotho expression in 60% of limited disease small cell lung cancer [14] while only in one third of large cell neuroendocrine carcinoma [15]. In the current study, although ANOVA showed significant difference between SCLC, ACA and SCC, the post-hoc test did not reveal difference between any two groups. There was no difference between any histological types and healthy subjects either, so we do not interpret the difference as clinically relevant. However, the study was powered only to the comparison of lung cancer patients and control subjects, not for subgroup analysis.

The results of Usuda et al., describing that klotho expression is predictive of a favorable outcome in both limited disease SCLC [14] and large cell neuroendocrine carcinoma [15] as well as the observation that miR-10b microRNA (which has an inverse correlation with survival) was negatively correlated with klotho in NSCLC [38] suggested that metastatic lung cancer will be characterized with lower klotho levels than less advanced stage disease. In vivo, we could not detect any difference between circulating klotho in stage I-III and stage IV disease.

Klotho has been suggested to serve as a therapeutic target of the disease based on different observations: Gene expression was associated with the resistance of lung cancer cells to cisplatin in vitro and in murine lung cancer model, klotho knockdown increased cisplatin resistance [18]. Furthermore, IGF1R inhibition can play a role in re-sensitizing lung cancer to certain drugs [39], thus klotho, an IGF1R inhibitor may have future therapeutic interest. As a second step, we aimed to continue our study to investigate whether platinum drug sensitivity might be predicted from initial klotho concentration, but being aware of the negative first step results, we decided not to proceed in this direction.

In conclusion, our results suggest that circulating klotho cannot serve as a potential biomarker in lung cancer. Further studies are warranted to examine the relation between lung tissue gene expression and circulating klotho levels, and to explore the detailed mechanism of action of klotho protein in lung cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the

institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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