Original Research

Novel interleukin-33 and its soluble ST2 receptor as potential serum biomarkers in parotid gland tumors

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Impact statement

Parotid gland tumors seem to be an increasingly important medical challenge, mostly due to a noticeable increase in the incidence. It would be crucial to find an easily determinable biomarker of tumor existence, its recurrence or malignant potential. We observed for the first time that serum IL-33 level was significantly elevated in patients with various types of parotid gland tumors and its sST2 receptor levels were significantly higher in pleomorphic adenoma and acinic cell carcinoma patients compared to the controls. We believe that our study helps to understand the biology of the tumors and a potential role of a relatively newly identified cytokine IL-33 in the pathophysiology of the parotid gland tumors.

Abstract

An increasing number of patients with parotid gland tumors have been observed in recent years. The relationship between the immune system and tumor formation is thoroughly investigated. However, newly discovered molecules offer a new insight into the pathophysiology of malignancies. It would be ideal to find an easily determinable biomarker of tumor existence, its malignant potential or a biomarker suggesting the probability of disease recurrence. Our study is the first to examine serum concentrations of IL-33 and its sST2 receptor in patients with various types of parotid gland tumors. Serum IL33, sST2, IL-4 and IL-10 concentrations were determined in patients with benign and malignant parotid gland tumors (pleomorphic adenoma, Warthin's tumor, myoepithelioma and acinic cell carcinoma). We observed for the first time that serum IL-33 level was significantly elevated in patients with various types of parotid gland tumors and sST2 levels were significantly higher in pleomorphic adenoma and acinic cell carcinoma patients compared to the controls. Our results demonstrate for the first time that serum IL-33 and its sST2 receptor may be important factors in the pathology of parotid gland tumors. Although our results are promising, further investigations are required to detect if serum concentrations of those molecules may be a biomarker in parotid gland tumors.

Keywords: Interleukin-33, sST2, tumor, parotid gland, salivary gland, pleomorphic adenoma

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Introduction

Currently, parotid gland tumors seem to be an increasingly important medical challenge, mostly due to a noticeable increase in the incidence.[1]

Although surgical procedures have been quite well known for about 70 years[2] and technical progress offers new tools, parotid gland surgery is still challenging.[2–4] Iatrogenic damage to the nerve results in partial or total paralysis of the mimic muscles of the face side, depending whether only one branch or the main trunk of the nerve was damaged, respectively (Figure 1). The rates of persistent VII nerve paralysis after benign tumor surgery vary from 0 to about 50%, depending on the operating center, the number of approaches, and the tumor size.[4,5] The minimally invasive surgical approach (e.g. extracapsular dissection or endoscopic approach) may be the optimal technique; however, it can be used by experienced surgeons in some limited cases only (small, mobile tumors of the superficial lobe).[2,3]

Interleukin-33 (IL-33), previously described as a nuclear factor from high endothelial venules (NF-HEVs),[6] is a relatively newly identified cytokine belonging to the interleukin-1 (IL-1) family of cytokines.[7,8] It is known as a dual function cytokine. It can be found in the nucleus of epithelial cells and fibroblasts where the cytokine is

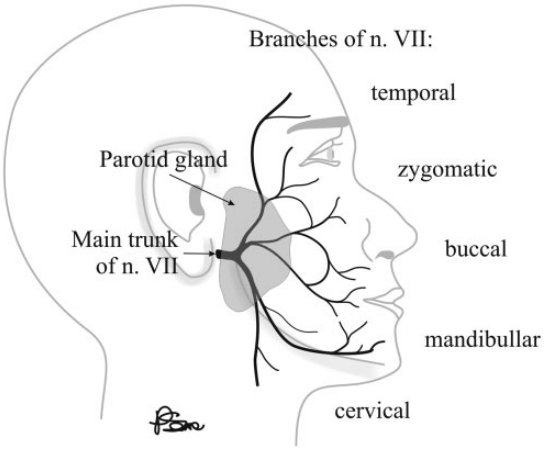

Figure 1. The anatomy of the facial nerve (VII).

produced and stored.[9,10] On the other hand, although the exact mechanism of the extracellular release is still unknown, it was found in the peripheral blood.[10] Currently it is known that some intracellular molecules such as IL-1 family cytokines together with IL-33 are released from necrotic cells and act as local inflammatory process activators – "alarmins."[9,11]

Two receptors bind soluble IL-33, i.e. IL-1RL1/ST2 (ST2) and IL-1 receptor accessory protein (IL-1RAP).[12] Based on *in vitro* studies, soluble form of ST2 was proposed to be a decoy receptor that attenuates the effects of IL-33.[13] It was proposed that sST2 may be a useful biomarker in cardiovascular diseases.[14,15] Although as mentioned, the soluble form of ST2 should have the sequestering impact on IL-33 actions, its role in tumor pathology seems unclear and the published reports seem to present its opposite impact depending on tumor histology[16,17] showing the need for investigation on that topic.

The overexpression of IL-33 or the IL-33/ST2 axis is proposed to be negatively correlated with the prognosis in several diseases such as breast,[18] lung,[19] gastrointestinal tract[20] or hepatic[21] cancers.

Head and neck squamous cell carcinoma (HNSCC) is a term describing the family of squamous cell cancers located in the region of the oral cavity, pharynx, larynx, and nose together with paranasal sinuses. It was proposed that cancer-associated fibroblasts (CAFs) release IL-33 and therefore are responsible for the ability for invasion and metastasis of HNSCC tumors.[22] Furthermore, high overexpression of IL-33 and IL-33/ST2 axis was proposed to be responsible for worse prognosis of the disease.[22,23] These data, however, need further investigation.

There is insufficient information on IL-33 and its receptors in salivary gland diseases.[24,25] In the experiment by Rössle *et al.*,[25] the overexpression of nuclear IL-33 was found in most benign and some malignant tumor tissues of salivary glands. Moreover, those authors reported that IL-33-positive malignant tumors revealed favorable histological parameters and the overall survival rate.

Taking together the above facts that IL-33 seems to play an important role in some neoplasms[26] and that the molecule was found in the tissue of salivary gland tumors,[25] we decided to further investigate the hypothesis previously proposed by Rössle *et al.* that IL-33 might be the biomarker of parotid gland tumors. Moreover, it was proposed that Th2 cells present a negative role in salivary gland tumors[27] and the activity of Th2 cells is regulated by IL-33.[23] Therefore, we examined the serum level of IL-33 in parotid gland tumor patients and controls. Furthermore, we decided to examine the serum concentration of its sST2 receptor.

Moreover, as suggested before, the action of IL-33 may be dependent on other cytokines, i.e. IL-4[28] or IL-10,[29] the cytokines, whose connection with cancers was previously proposed.[30–33] We decided to additionally examine serum levels of those cytokines in our patients.

It would be crucial to find an easily determinable biomarker of tumor existence, its recurrence or malignant potential. Therefore, patients with the presence of such a marker would undergo more frequent imaging and control examination. Thus, the potential malignancy or recurrence would be diagnosed earlier and treatment could be provided in advance. Moreover, some new therapeutic options might appear.

So far only single reports have been published on the role of IL-33 and its sST2 receptor in salivary gland tumors.[25,34] Our prospective study is the first to examine serum concentrations of IL-33 and its soluble sST2 receptor in patients with various types of parotid gland tumors.

We hope that our study will contribute to a better understanding of the pathophysiology of parotid gland tumors.

Material and methods

Patients

We examined prospectively 108 patients with parotid gland tumor who were admitted to the Department of Otorhinolaryngology and Oncological Laryngology in Zabrze, Medical University of Silesia in Katowice, Poland. To eliminate differences of the measured factors depending on age and sex, we matched the selected groups to the least populated group, i.e. acinic cell carcinoma (ACC) group. Therefore, 17 patients with pleomorphic adenoma (PA group), 12 patients with Warthin's tumor (WT group), 11 with myoepithelioma (Myo group) and 10 with ACC (ACC group) were enrolled in the study. The control group consisted of 30 healthy sex- and age-matched individuals admitted to our Department for nasal septum surgery, not involving rhinosinusitis or other laryngological conditions. Patients with previous oncological treatment, diabetes mellitus, obesity, other metabolic disorders or under cardiac treatment were excluded from the study (Table 1).

The type of surgery in the examined groups was determined according to the suggestions of the European Salivary Gland Society.[35] In the ACC group, two patients underwent superficial parotidectomy (Par I-II) as a consequence of a benign tumor diagnosed in fine-needle biopsy.

Biochemical analysis

Venous blood samples (2 mL) obtained from patients (Vacuette tubes, Greiner Bio-One GmbH, Austria) were immediately centrifuged, frozen and stored in −80°C

Table 1. Demographic characteristics of the examined groups.

Examined groups	Control ($n = 30$)	PA ($n = 17$)	WT ($n = 12$)	Myo ($n = 11$)	ACC ($n = 10$)
Age (years±SD)	55.5±9.4	53.5±11.4	57.9±6.5	52.9±14.2	59.9±8.2
Female/male	20/10	11/6	9/3	7/4	7/3
Surgery type					
ECD	NA	1	0	0	0
Par I or II		3	6	7	0
Par I-II		11	2	2	2
Par I-III		2	4	2	0
Par I-IV		0	0	0	8
Mean tumor size (mm) (min-max)	NA	26 (13–55)	25 (12–40)	23 (15–28)	24 (18–30)
Tumor side right/left	NA	13/4	8/4	9/2	4/6

PA: pleomorphic adenoma group; WT: Warthin's tumor group; Myo: myoepithelioma group; ACC: acinic cell carcinoma group; ECD: extra-capsular dissection; Par: parotidectomy; NA: non-applicable.

until the final examination. Serum IL33, sST2, IL-4 and IL-10 concentrations were determined using ELISA methods (R&D, USA, Elx 800 device – BIO-TEK Instruments, USA). The concentrations of CRP were determined using standard methods[36] (Cobas 600 analyzer, Roche, USA).

Statistical analysis

The statistical analysis was performed using Statistica 13 (StatSoft), PQStat (PQStat Software) and Excel (Office, Microsoft) software. The normality of the distribution of the results was tested using the Shapiro–Wilk test. The U Mann–Whitney test or the Kruskal–Wallis test for quantitative features was used as a detection test for the significance of differences between groups. We used the Mann–Whitney test as the *post hoc* test for the Kruskal–Wallis test. The correlations were performed by the Pearson method. To improve our results, we used receiver operating characteristics (ROCs) curve analysis in selected cases. Moreover, the area under the curve (AUC) was calculated using the de Long method. The level of significance was established as $P < 0.05$.

The experimental protocol was approved by the local ethical committee of the Medical University of Silesia in Katowice No KNW/022/KB1/106/16 and No KNW/0022/KB1/106/I/16/17.

Results

Serum IL-33 concentrations

Our results revealed elevated serum levels of IL-33 in all the analyzed types of parotid gland tumors (PA, WT, Myo and ACC) as compared to the control group. The highest level of IL-33 was observed in the Myo group (6.363 ± 0.639 pg/mL). A statistically similar level of that cytokine was also noted in the WT group (5.771 ± 0.752 pg/mL; $P = 0.07$). Both mean IL-33 levels in Myo and WT patients were significantly higher compared not only to the control group (4.359 ± 0.492 pg/mL; $P = 0.000001$ in both) but also to the PA group (5.057 ± 0.506 pg/mL; $P = 0.00001$ and $P = 0.009$, respectively) (Figure 2). Moreover, in the ACC group, the serum level of IL-33 (5.601 ± 0.517 pg/mL) was significantly lower compared to Myo and higher compared to the PA group ($P = 0.009$ and $P = 0.017$, respectively). Furthermore, we observed that maximum tumor size strongly positively correlated with IL-33 level in WT patients ($r = 0.72$; $P < 0.05$). Moreover, in malignant tumors (ACC group), IL-33 levels correlated very strongly positively with IL-10 concentrations ($r = 0.996$, $P < 0.05$). To evaluate the diagnostic performance of serum IL-33, we used ROC curve tests. It could be observed that the value of 4.7 pg/mL of serum IL-33 concentration may be considered the cut-off point with sensitivity and specificity of 0.96 and 0.82, respectively. Moreover, the AUC of this test achieved the value of 0.93 (Figure 3).

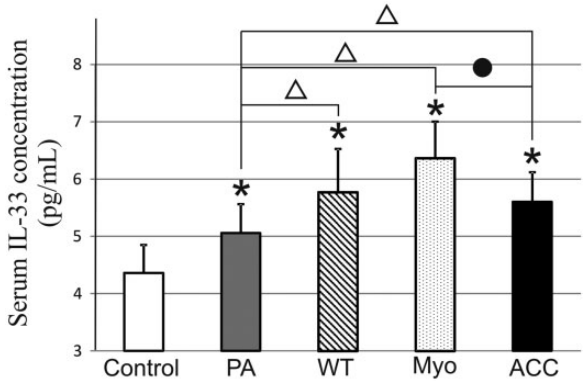

Figure 2. Serum IL-33 concentrations (pg/mL) in PA – pleomorphic adenoma group, WT – Warthin's tumor group, Myo – myoepithelioma group, ACC – acinic cell carcinoma group. *$P<0.05$ versus Control group; Δ$P<0.05$ versus PA group; •$P<0.05$ versus Myo group. Data are presented as mean±SD.

Serum sST2 concentrations

We found that sST2 serum concentrations in our tumor patients were elevated in the PA group (3456.9 ± 1409.1 pg/mL) and the ACC group (3099.1 ± 560.6 pg/mL) versus the control group (2018.7 ± 873 pg/mL; $P = 0.0001$ and $P = 0.0008$, respectively). Moreover, we noted a significant difference in sST2 concentration between PA versus WT and Myo patients (3456.9 ± 1409.1 versus 2181.6 ± 764.9 pg/mL; $P = 0.01$ and 2150.7 ± 425.9 pg/mL; $P = 0.009$). Similar differences between ACC versus WT and Myo groups were observed ($P = 0.006$ and $P = 0.0005$, respectively) (Figure 4). Serum levels of this receptor in WT and Myo groups were similar to the control. Furthermore, we observed that maximum tumor size

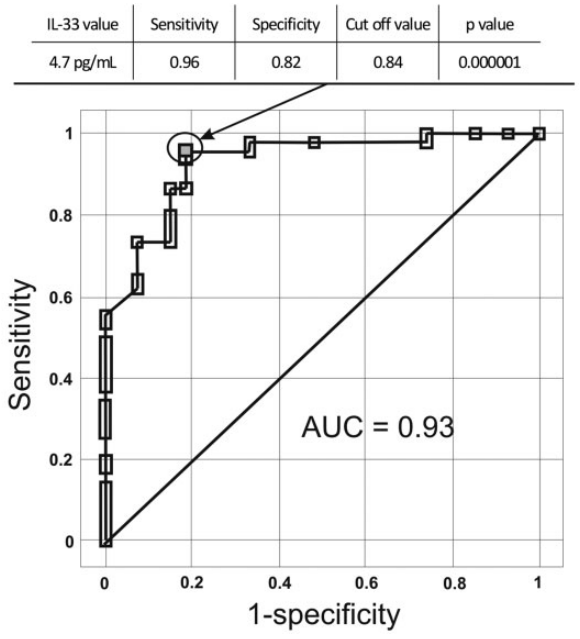

Figure 3. The results of the ROC curve analysis for serum IL-33 concentration at the cut-off point of 4.7 pg/mL. AUC: area under curve; $P < 0.000001$. (A color version of this figure is available in the online journal.)

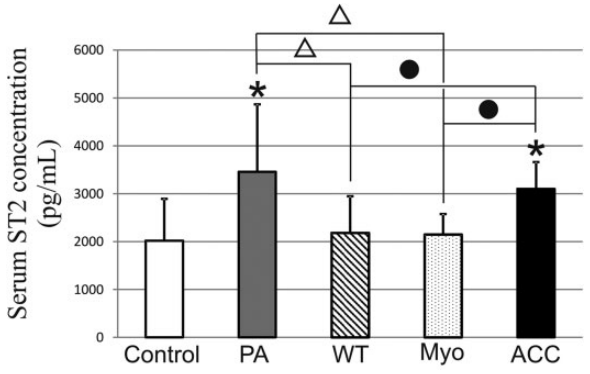

Figure 4. Serum concentrations of the soluble form of IL-33 ST2 receptor (sST2) (pg/mL) in PA – pleomorphic adenoma group, WT – Warthin's tumor group, Myo – myoepithelioma group, ACC – acinic cell carcinoma group. *$P < 0.05$ versus Control group; Δ$P < 0.05$ versus PA group; •$P < 0.05$ versus ACC group. Data are presented as mean ± SD.

strongly positively correlated with sST2 level in WT patients ($r = 0.796$; $P < 0.05$).

Serum IL-4 concentrations

Although in all patients with parotid gland tumor, the mean concentration of IL-4 was higher than in the controls, the statistical difference was observed only in the ACC group (25.1 ± 0.5 versus 25.7 ± 0.6 pg/mL; $P = 0.03$) (Figure 5). Moreover, a moderate positive correlation between serum IL-4 and IL-10 levels in PA patients ($r = 0.523$, $P < 0.05$) was found.

Serum IL-10 concentrations

The mean IL-10 levels of the investigated tumor patients were slightly but insignificantly higher than in the controls.

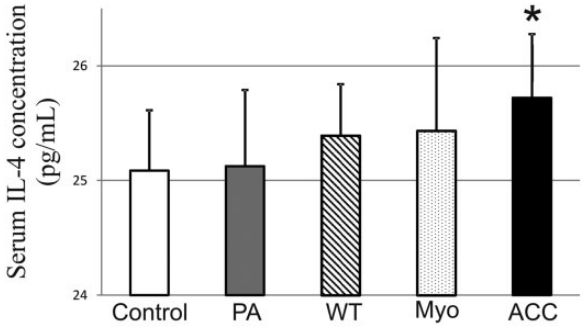

Figure 5. Serum IL-4 concentrations (pg/mL) in PA – pleomorphic adenoma group, WT – Warthin's tumor group, Myo – myoepithelioma group, ACC – acinic cell carcinoma group. *$P<0.05$ versus Control group. Data are presented as mean±SD.

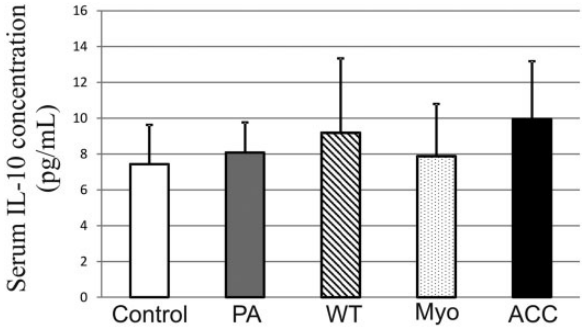

Figure 6. Serum IL-10 concentrations (pg/mL) in PA – pleomorphic adenoma group, WT – Warthin's tumor group, Myo – myoepithelioma group, ACC – acinic cell carcinoma group. Data are presented as mean ± SD. $P < 0.05$.

Therefore, we did not observe any statistical differences in IL-10 levels between the investigated groups (Figure 6). However, a moderate positive correlation between serum IL-10 and IL-4 levels in PA patients ($r = 0.523$, $P < 0.05$) was found. Furthermore, in malignant tumors (ACC group), IL-33 levels correlated very strongly positively with IL-10 concentrations ($r = 0.996$, $P < 0.05$).

Serum CRP concentrations

In all our subjects, CRP concentration was below 5.0 mg/L. However, some statistical differences were observed. In the PA group, CRP level was significantly higher compared to the controls (4.31 ± 0.89 vs. 2.11 ± 1.28 mg/L; $P = 0.008$). Moreover, the CRP level in the Myo group was also higher than in PA and WT patients (1.74 ± 0.89 mg/L, $P = 0.0003$ and 1.77 ± 1.33 mg/L, $P = 0.02$, respectively). The level of CRP in ACC patients did not differ from the controls, PA, WT or Myo individuals (Figure 7).

Discussion

There have been an increasing number of patients with parotid gland tumors observed in recent years (approximately a 2-fold annual increase in our department).[1] Although there are some theories on possible indicators (such as mobile phones), no certain evidence has been presented as yet.[37,38] It is obvious that early surgical procedure would be safer in the majority of parotid gland tumors and

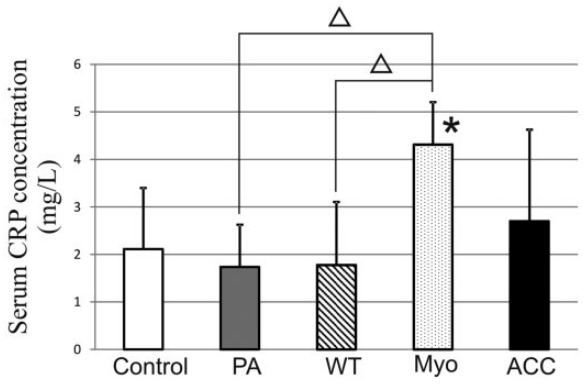

Figure 7. Serum CRP concentrations (mg/L) in PA – pleomorphic adenoma group, WT – Warthin's tumor group, Myo – myoepithelioma group, ACC – acinic cell carcinoma group. *$P<0.05$ versus Control group; Δ$P<0.05$ versus Myo group. Data are presented as mean±SD.

proper surgical qualification is therefore critical. Until now there has been no serum biomarker for these tumors. Consequently, finding an easily determinable serum biomarker would be valuable. Since IL-33 and sST2 were shown to have an important impact on many neoplasms,[23] we decided to investigate them also in our parotid gland patients as a potential serum biomarkers of those tumors.

The abundant expression of IL-33 was found in epithelial cells of blood vessels in several human tumors.[9] Moreover, an increased serum concentration of the cytokine was found in other pathologies and tumor diseases.[39,40] Therefore, we anticipated that parotid gland tumors might be the source of IL-33 circulating in the blood.

Intreleukin-33 is a relatively newly identified cytokine belonging to the IL-1 family. There are only a few available papers concerning this molecule in salivary gland pathologies.[24,25] We observed for the first time that serum IL-33 level was significantly elevated in patients with various types of parotid gland tumors (pleomorphic adenoma, Warthin's tumor, myoepithelioma and ACC) compared to healthy controls. These results are consistent with the observations of Rössle *et al.*[25] Unlike our study, their report was based on histological examination of IL-33 presence in the tissues of salivary gland tumors. However, not every tumor tissue revealed the activity of IL-33.[25] Determination of serum IL-33 level is the subject of interest in many oncological experiments. The elevated serum level of IL-33 was also observed in different carcinomas[21,40,41] and the overexpression of IL-33 in carcinoma-associated fibroblasts of HNSCC was associated with a poor clinical outcome.[22] This cytokine may act as an "alarmin" although its precise role still remains unclear.[9] Our results suggest that serum IL-33 concentration may be considered the biomarker of the tumor presence (AUC = 0.93) with the value of 4.7 pg/mL as the cut-off point. We may not be certain whether the tumor would be of benign or malignant origin, however.

Signal transducer and activator of transcription (STAT) is a term defining a family of proteins that transmit signals to specific DNA transcription promoters and thus regulate gene expression.[42] STATs were found in a normal parotid gland tissue as well as in some salivary gland tumors.[43] A different distribution of STAT in selected tumors may at least partially explain their nature. It was previously shown that some oncogenes and oncogenic microRNA are strongly overexpressed in PA.[44] Moreover, Andreasen *et al.*[44] showed that activation of IL-6/Janus kinase/STAT3 pathway is responsible for tumorigenesis in PA and that activation was independent on HER2 and EGFR receptors. Thus, other receptor pathways should be involved. Considering our results and those of Andreasen *et al.*, we anticipate that IL-33 ST2-dependent pathway is a potential way to activate STAT3-mediated gene regulation.

We propose that IL-33 released to blood in the case of stress situations such as injury or inflammation[45] via ST2 receptors may activate Th2 cells that release IL-6[46] and therefore activate the above STAT3-dependent pathway of tumorigenesis. However, such a hypothesis of tumor initiation by IL-33 needs to be supported in *in vitro* studies.

Some parotid gland tumors present with locally increased inflammatory reaction.[47] Studies showed that a prominent lymphoid infiltrate is associated with ACC tumors.[48] Similarly, a heavy mixed inflammatory infiltrate with an increased number of CD4 cells was observed in WT.[49,50] Those results supported by our findings of an increased serum IL-33 in tumor patients may predict that parotid gland tumors might be the source of the circulating cytokine. Moreover, the fact that all our patients with histologically different parotid gland tumors had elevated serum IL-33 concentrations suggests that IL-33 may play an important role in the pathology of tumors in this tissue. However, the precise role of IL-33 and its sST2 receptor needs further *in vitro* and *in vivo* investigations.

There are two target receptors for IL-33, i.e. IL-1RAP and ST2. While L-1RAP is a membrane receptor, ST2 has its soluble form – sST2.[13] The soluble form of the receptor was found to have inhibitory effects on IL-33 and has already been extensively examined in cardiology[51] and is still under investigation in several groups of cancers.[16,17] Therefore, we decided to examine the serum level of sST2 in our patients. We observed significantly higher serum sST2 levels in PA and ACC patients as compared to the controls. The IL-33/sST2 axis is found to be important in tumorigenesis, since it was shown that sST2 inhibited IL-33-induced tumor angiogenesis and influenced tumor microenvironment.[17] In our results, we could observe that the groups of patients with high serum IL-33 concentration (WT and Myo) presented a low level of sST2 although we did not observe important correlations. This observation seems to support the previous reports according to which sST2 may attenuate the effects of IL-33 acting as a decoy receptor.[13] Based on our study results on IL-33 and sST2, we confirm this theory also in the case of parotid gland tumors.

Our observations revealed, however, that serum IL-33 and sST2 do not allow for differentiation between malignant and benign parotid gland tumors.

Additionally, we observed that the tumor size positively correlated with both IL-33 and sST2 serum concentrations in WT patients. This observation may be related to the previously described inflammatory reaction present in WT.[50] The autocrine release of IL-33 in the tumor microenvironment may be the source of the increase in circulating

cytokine levels related to tumor growth. On the other hand, an increased concentration of sST2 might be a physiological response or a pathological mechanism influencing the Th2-indicated immunologic status.[52] These considerations, however, need further investigation both in *in vitro* and clinical studies.

Moreover, we planned to examine additional cytokines with potential tumor involvement, i.e. IL-4 and IL-10. Unlike in the case of the previously reported squamous cell carcinoma, we did not observe differences in the plasma level of IL-10 in any of our investigated groups.[30] However, we observed a higher plasma level of IL-4 in ACC patients. Further investigations are required to assess whether anti-IL-4 treatment may have a similar impact as was proposed in thyroid cancer.[32] A strong positive correlation between IL-33 and IL-10 was observed in ACC patients. We anticipate that gathering a larger group of ACC patients may reveal more information about this type of tumor and it may shed a new light on malignant transformation of some benign salivary gland tumors.

Over 150 years ago, Rudolf Virchow observed that inflammatory cells are present in the neoplastic tissue and established a link between inflammation and neoplasms.[53] We believe that our results support this long-lasting hypothesis also in the case of salivary gland tumors. Similarly, we previously reported the whole-body oxidative stress in parotid gland tumor patients.[54] Confirmation of this hypothesis may also be the fact that in clinical practice we can often see cases of inflamed salivary gland tumors.[55,56] To conclude, we are of the opinion that among benign tumors, Myo and WT show a higher inflammatory component of the disease compared to PA. Moreover, ACC tumors of parotid glands demonstrate a high inflammatory potential.

The present work is a part of the series of studies performed by our team previously presenting the antioxidative status, adipocytokines and some interleukins in salivary gland tumors.[54,57] A narrow panel of the studied inflammatory molecules is the limitation of this study regarding the aspect of "tumor and inflammation." Further exploration of the issue of inflammation in salivary gland tumors other supported by histological investigations and blood tests would be useful. Additionally, extensive immunological examinations related to other cytokines, e.g. TNF-α would improve our knowledge on possible inflammatory processes in tumor environment.

Although further investigations are required, IL-33 and sST2 may be potential biomarkers in treatment of parotid gland tumors and the follow-up. It should, however, be pointed out that due to a wide histological and oncological variety of primary salivary gland tumors, finding one universal biomarker for all of those tumors seems difficult to achieve.

The IL-33/sST2 can also be found in saliva[58] and urine.[59] Although saliva is much easier to collect compared to blood samples, the levels of IL-33 and its receptors in saliva are, however, strongly dependent on local oral conditions.[60] Therefore, the salivary level of IL-33/sST2 seems difficult for the independent assessment. Nevertheless, further investigation regarding salivary and urinary levels of IL-33 or other cytokines seems interesting in the survey of tumors. We could not find any reports mentioning such investigation in cancer patients in the current literature.

The potential role of IL-33 and sST2 as biomarkers of recurrence in parotid gland tumors is currently being under investigation by our team. Perhaps the IL-33/sST2 axis plays a role in both promotion of tumorigenesis and, on the other hand, further tumor progression via a positive loop.

Conclusions

Our results demonstrate for the first time that serum IL-33 and its sST2 receptor may be important factors in the pathology of parotid gland tumors. Although our results are promising, further investigations are required to detect whether serum concentrations of those molecules may be a biomarker in parotid gland tumors. Therefore, the IL-33/sST2 axis is an interesting aim of investigation in the pathology and treatment of benign and malignant salivary gland tumors.

Authors' contributions: SP and ASM conceived and designed the study; SP, ZM and MB acquisitioned the data;

SP and ASM analyzed data and interpreted the results; SP and ASM created the figures and drafted the manuscript; SP, MM, MB and ASM edited and revised manuscript.

All authors approved the final version of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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