Prognostic impact of activin subunit inhibin beta A in gastric and esophageal adenocarcinomas

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Abstract
Purpose: Adenocarcinomas of the esophagus (AEG) and stomach (AS) are among the most common cancers worldwide. Novel markers for risk stratification and guiding treatment are strongly needed. Activin is a multi-functional cytokine with context specific pro- and anti-tumorigenic effects. We aimed to investigate the prognostic role of activin tumor protein expression in AEG/ASs.

Methods: Tissue from a retrospective cohort of 277 patients with AEG/AS treated primarily by surgery at the Charité - Universitätsmedizin Berlin was collected and analyzed by immunohistochemistry using a specific antibody to the activin homodimer inhibin beta A. Additionally, we evaluated T-cell infiltration and PD1 expression as well as expression of PD-L1 by immunohistochemistry as possible confounding factors. Clinico-pathologic data were collected and correlated with activin protein expression.

Results: Out of 277 tumor samples, 72 (26.0%) exhibited high activin subunit inhibin beta A protein expression. Higher expression was correlated with lower Union for International Cancer Control (UICC) stage and longer overall survival. Interestingly, activin subunit expression correlated with CD4+ T-cell infiltration, and the correlation with higher overall survival was exclusively seen in tumors with high CD4+ T-cell infiltration, pointing towards a role of activin in the tumor immune response in AEG/ASs.

Conclusion: In our cohort of AEG/AS, higher activin subunit levels were correlated with longer overall survival, an effect exclusively seen in tumors with high CD4+ cell infiltration. Further mechanistic research is warranted discerning the exact effect of this context specific cytokine.

Keywords: Gastric adenocarcinoma, Esophageal adenocarcinoma, Activin, INHBA, TGF-β superfamily

Introduction
Adenocarcinomas of the esophagus (AEG) and stomach (AS) are among the most common cancers worldwide [1]. Despite slowly falling incidence rates of AS in developed countries most probably due to lower H. pylori infection rates [2], AEGs are diagnosed more frequently, coinciding with an increase in Barrett’s esophagus [3]. Albeit therapeutic advances, prognosis remains dire especially in a metastasized setting with 5-year survival rates around 5% [1]. Novel markers for risk stratification and guiding treatment are strongly needed.

Activin, a transforming growth factor-beta (TGF-β) superfamily member is a multifunctional cytokine with a well-recognized role in the tumorigenesis of
gastrointestinal tumors [4]. Initially described as an upstream effector of follicle stimulating hormone [5], additional roles of activin were shown in contexts as inflammation [6–9], cancerogenesis [4, 10, 11] and cancer cachexia [12]. The pleiotropic and context-specific effects of the activin pathway can be partly understood through the complexity of activin signaling. Activin ligand consist of two Inhibin beta subunits with several isoforms, the most abundant and best described being activin A, consisting of two Inhibin beta A subunits. Besides the homodimer, a heterodimer of Inhibin beta A and Inhibin alpha exists. For the sake of readability, activin will be used in this manuscript in lieu of activin homodimer subunit beta A. It binds to its type 2 receptor which in turn dimerizes and phosphorylates the activin type 1 receptors [13], leading to activation of so-called canonical SMAD-dependent and numerous non-canonical pathways, including the PI3K, MAP/ERK and NFkappaB pathways [14, 15].

In tumorigenesis, pro and anti-oncogenic functions are documented. In early stage colorectal cancer, activin acts similarly to TGF-β growth-inhibiting through SMAD-dependent signaling. In later stages, activin acts primarily pro-metastatic through non-canonical pathways [16]. Additionally, first reports on a role of activin in the tumor immune response are emerging. The literature describes pro- and anti-inflammatory effects of activin, which are also characteristic for other TGF-beta superfamily members [17]. Initially best characterized for strictly pro-inflammatory effects on the innate immune system, especially neutrophils [18], new data point to an immune modulating function of activin signaling with context specific pro- and anti-inflammatory effects. Specifically, activin was shown to induce CD4+ regulatory T-cells both in the context of inflammatory conditions such as allergic airway disease [19] and in the context of cancer. Strikingly, both pro- and anti-tumor genic effects of activin through CD4 cells were described in the literature. In the context of breast and skin cancer, activin was shown to facilitate immune evasion through its action on CD4+ regulatory T-cells [20]. Recently, activin was shown to drive differentiation of CD4+ cells into Th17 cells [21].

Data on activins role in the context of AEG/AS are sparse. Multiple investigators have shown a growth-inhibitory and pro-apoptotic function of activin in gastric cancer cell lines [22, 23]. Additionally, and consistent with activins dual role in other tumors such as colorectal cancer, mRNA silencing of activin leads to reduced cell migration and invasion in vitro [24]. Data from two Japanese cohorts demonstrate higher activin mRNA-expression correlating with worse prognosis and shorter overall survival in patients after primary resection [25] or after adjuvant chemotherapy and resection [26], respectively. Additionally, in a Chinese cohort from patients with AEG/AS, Wang et al. reported a correlation between protein expression of the activin subunit inhibin beta A and shorter overall survival [27]. As the five-year survival rate in this cohort was around 80%, the transferability of these results is questionable.

Overall, the in vitro data show an anti-oncogenic effect, but the cohort data show a positive correlation with poor survival. Despite its known immune modulating effects [28] correlation of activin expression and tumor infiltrating lymphocytes in AEG/ASs are missing.

The aim of this study was to investigate the prognostic role of activin subunit inhibin beta A in a large Caucasian AEG/AS cohort and to test a potential correlation of activin expression and level of lymphocyte infiltration.

**Material & Methods**

**Cohort**

Clinical data from patients with AGE/AS of all tumor stages, primarily treated by surgery between 1992 and 2004 at the Charité - Universitätsmedizin Berlin, were collected retrospectively. From 2004 onwards, patients were treated with (neo)-adjuvant chemotherapy at our institution. As the aim of this study was to investigate the prognostic impact of activating subunit inhibin beta A and for the sake of comparability, we specifically chose a cohort exclusively treated by surgery. The mean follow-up was 113.6 months (95% CI: 103.9–123.2). Overall survival was defined as time from diagnosis to death or last follow-up. Disease-specific survival was defined as time from diagnosis to tumor-related death or last follow-up. The data including patient characteristics and follow-up information were retrieved from the patient management software (SAP®) and the regional population-based cancer registry ("Gemeinsames Krebsregister") and are summarized in Table 1. All patients included in this study gave their informed consent prior to their inclusion. This study was approved by the Institutional Review Board of the Charité (EA4/115/10).

**Tissue samples**

Tissue samples were collected from the archive of the Institute of Pathology, Charité - Universitätsmedizin Berlin. Paraﬃn embedded tumor samples (n = 277) were available from surgically treated chemotherapy-naive patients. All samples were reevaluated according to histological diagnosis, tumor stage and grade, and classified by the histological architecture of AGE/S carcinoma using Lauren’s and Ming classiﬁcation by a specialist for gastrointestinal pathology (M.W.). Data concerning tumor size, depth of invasion, and tumor invasion of veins or lymphatic vessels were retrieved from the
Table 1  Patient characteristics of the analyzed patient cohort and distribution of Activin high and low expressing primary tumors. Significance calculated by X²-Test

|                      | All           | Activin    |        |        |        |        |        |
|----------------------|---------------|------------|--------|--------|--------|--------|--------|
|                      | n (%)         | low. n (%)  | high. n (%) | p     |        |        |        |
| Gender               |               |            |        |        |        |        |        |
| Female               | 100 (36.1)    | 78 (78.0)  | 22 (22.0) | 0.298 |        |        |        |
| Male                 | 177 (63.9)    | 128 (72.3) | 49 (27.7) |        |        |        |        |
| Age Group            |               |            |        |        |        |        |        |
| < 65 years           | 156 (56.3)    | 110 (70.5) | 46 (29.5) | 0.095 |        |        |        |
| >= 65 years          | 121 (43.7)    | 96 (79.3)  | 25 (20.7) |        |        |        |        |
| Localization         |               |            |        |        |        |        |        |
| Gastric Cancer       | 230 (83.0)    | 171 (74.3) | 59 (25.7) | 0.986 |        |        |        |
| AEG                  | 47 (17.0)     | 35 (74.5)  | 12 (25.5) |        |        |        |        |
| Tumor stage          |               |            |        |        |        |        |        |
| T1                   | 38 (13.7)     | 21 (55.3)  | 17 (44.7) | 0.041 |        |        |        |
| T2                   | 105 (37.9)    | 78 (74.3)  | 27 (25.7) |        |        |        |        |
| T3                   | 102 (36.8)    | 80 (78.4)  | 22 (21.6) |        |        |        |        |
| T4                   | 31 (11.2)     | 26 (83.9)  | 5 (16.1)  |        |        |        |        |
| unspecified          | 1 (0.4)       | 0 (0.0)    | 1 (100.0) |        |        |        |        |
| Node Stage           |               |            |        |        |        |        |        |
| N0                   | 71 (25.6)     | 45 (63.4)  | 26 (36.6) | 0.014 |        |        |        |
| N+                   | 206 (74.4)    | 161 (78.2) | 45 (21.8) |        |        |        |        |
| Distanter Metastasis |               |            |        |        |        |        |        |
| M0                   | 196 (70.8)    | 136 (69.4) | 60 (30.6) | 0.003 |        |        |        |
| M1                   | 81 (29.2)     | 70 (86.4)  | 11 (13.6) |        |        |        |        |
| Lymphatic vessel invasion |     |            |        |        |        |        |        |
| L0                   | 92 (33.2)     | 63 (68.5)  | 29 (31.5) | 0.137 |        |        |        |
| L1                   | 165 (59.6)    | 127 (77.0) | 38 (23.0) |        |        |        |        |
| unspecified          | 20 (7.2)      | –          | –        |        |        |        |        |
| Vein invasion        |               |            |        |        |        |        |        |
| V0                   | 164 (59.2)    | 116 (70.7) | 48 (29.3) | 0.107 |        |        |        |
| V1                   | 90 (32.5)     | 72 (80.0)  | 18 (20.0) |        |        |        |        |
| unspecified          | 23 (8.3)      | –          | –        |        |        |        |        |
| Grading              |               |            |        |        |        |        |        |
| G1                   | 1 (0.4)       | 0 (0.0)    | 1 (100.0) | 0.35  |        |        |        |
| G2                   | 73 (26.4)     | 48 (64.9)  | 26 (35.1) |        |        |        |        |
| G3                   | 200 (72.2)    | 157 (78.5) | 43 (21.5) |        |        |        |        |
| unspecified          | 3 (1.1)       | –          | –        |        |        |        |        |
| Lauren Classification |               |            |        |        |        |        |        |
| Intestinal           | 98 (35.4)     | 65 (66.3)  | 33 (33.7) | 0.114 |        |        |        |
| Diffuse              | 139 (50.2)    | 108 (77.7) | 31 (22.3) |        |        |        |        |
| Mixed                | 37 (13.4)     | 31 (83.8)  | 6 (16.2)  |        |        |        |        |
| unspecified          | 3 (1.1)       | –          | –        |        |        |        |        |
| Ming Classification  |               |            |        |        |        |        |        |
| expansive            | 109 (39.4)    | 72 (66.1)  | 37 (33.9) | 0.034 |        |        |        |
| infiltrative         | 165 (59.6)    | 132 (80.0) | 33 (20.0) |        |        |        |        |
| unspecified          | 3 (1.1)       | –          | –        |        |        |        |        |
| MMR                  |               |            |        |        |        |        |        |
| proficient           | 240 (86.6)    | 178 (74.2) | 62 (25.8) | 0.997 |        |        |        |
| deficient            | 31 (11.2)     | 23 (74.2)  | 8 (25.8)  |        |        |        |        |
Tissue samples were screened in a HE-stained section for representative areas of solid tumors. Two 1 mm-diameter tissue cores were punched out from each of the 277 available cases and were transferred to a recipient paraffin block. After re-melting, sections (4 μm thick) were consecutively cut from each tissue microarray block (TMA). HE staining was performed on TMA sections for reconfirmation of content of tumor and non-tumor tissue in each core.

Immunohistochemical analysis of expression of CD3⁺, CD4⁺ and CD8⁺, as well as PD-1 and PD-L1 was performed on consecutive TMA sections using a specific monoclonal antibody as previously described. Correlation of prognosis with these factors in this cohort as well as representative images of the stainings were previously published [29].

Immunohistochemical staining for activin homodimer subunit inhibin beta A was performed manually as previously described [30]. Briefly, paraffin sections were dewaxed in xylene (2 × 10 minutes at room temperature) and hydrated in a descending alcohol series (5 minutes at room temperature each: 100%/90%/70%/30%/0% ethanol). Epitopes were retrieved heat-induced in a pressure cooker at pH 8 (0.5 M EDTA buffer). After rinsing, sections were incubated with anti-Inhibin beta A from ANSH labs Webster, Texas, US (clone AI006, dilution 1:1500) for 30 minutes at room temperature. For detection, the Dako Universal LSAB2 Kit was used (Agilent). Nuclei were counterstained with hematoxylin (Merck) and slides were coverslipped with glycerol gelatin (Merck). Primary antibody was omitted in negative controls.

### Table 1 (continued)

|                  | All                      | Activin                  | Activin                  | p    |
|------------------|--------------------------|--------------------------|--------------------------|------|
|                  | Total                    | low.                     | high.                    |      |
|                  | n (%)                    | n (%)                    | n (%)                    |      |
| unspecified      | 6 (2.2)                  | –                        | –                        | –    |
| CD3              |                          |                          |                          |      |
| low              | 137 (49.5)               | 105 (76.6)               | 32 (23.4)                | 0.691|
| high             | 118 (42.6)               | 85 (72.0)                | 33 (28.0)                |      |
| unspecified      | 22 (7.9)                 | –                        | –                        | –    |
| CD4              |                          |                          |                          |      |
| low              | 147 (60.0)               | 120 (81.6)               | 27 (18.4)                | 0.009|
| high             | 98 (35.4)                | 63 (64.3)                | 35 (35.7)                |      |
| unspecified      | 32 (11.6)                | –                        | –                        | –    |
| CD8              |                          |                          |                          |      |
| low              | 134 (48.4)               | 106 (79.1)               | 28 (20.9)                | 0.217|
| high             | 120 (43.3)               | 84 (70.0)                | 36 (30.0)                |      |
| unspecified      | 23 (8.3)                 | –                        | –                        | –    |
| PD-L1 in lymphocytes |              |                          |                          |      |
| negative         | 182 (65.7)               | 141 (77.5)               | 41 (22.5)                | 0.065|
| positive         | 81 (29.2)                | 54 (66.7)                | 27 (33.3)                |      |
| unspecified      | 11 (4.0)                 | –                        | –                        | –    |
| PD-L1 in tumor cells |                |                          |                          |      |
| negative         | 245 (88.4)               | 185 (75.5)               | 60 (24.5)                | 0.062|
| positive         | 18 (6.5)                 | 10 (55.6)                | 8 (44.4)                 |      |
| unspecified      | 11 (4.0)                 | –                        | –                        | –    |
| PD-1 in lymphocytes |              |                          |                          |      |
| negative         | 112 (40.4)               | 90 (80.4)                | 22 (19.6)                | 0.051|
| positive         | 145 (52.3)               | 101 (69.7)               | 44 (30.3)                |      |
| unspecified      | 20 (7.2)                 | –                        | –                        | –    |
| PD-1 in tumor cells |                |                          |                          |      |
| negative         | 259 (93.5)               | 192 (74.1)               | 67 (25.9)                | –    |
| positive         | 0 (0.0)                  | 0 (0)                    | 0 (0)                    | –    |
| unspecified      | 18 (6.5)                 | –                        | –                        | –    |
control section. Pictures were acquired via the Vectra 3 Automated Quantitative Pathology Imaging System (Akoya Bioscience) employing Vectra3 (Akoya Bioscience, version 3.0.7). Examples for representative staining are shown in Fig. 1. All cases were scored by two blinded investigators by assessing two cores per tumor sample. Both the proportion of positive tumor cells (<25%;0 25–50%;1 50–75%;2 >75%;3) and the staining intensity was assessed (intensity score 1–4, with staining intensity corresponding to: 1: no staining, 2: weak staining 3: moderate staining and 4: strong staining.). Subscores were multiplied to calculate final score from 0 to 12, with a score >6 interpreted as activin high expression. In the case of disagreement in scoring between the two investigators (JJS and CT), cases were reassessed and discussed. Additionally, staining pattern and scoring was reevaluated and independently confirmed by a pathologist (AA). For a detailed version of the visualizing agents, see Supplemental Table S1.

Statistics
Statistical analysis was performed using IBM SPSS Version 24. Overall survival was evaluated in months from time of diagnosis until death or until the most recent follow-up using Kaplan–Meier plots. Associations of activin expression with tumor size, distant and lymph node metastasis, venous and lymphatic infiltration, Lauren and Ming classification, grading and UICC classification were tested by the X2 test. Univariate survival analyses were performed according to the Kaplan-Meier method with the log-rank test for assessment of statistical significance. An alpha-level of 0.05 was set prior to all experiments.

Results
Clinical characteristics
Data of 277 Patients (detailed clinico-pathological characteristics are summarized in Table 1) were analyzed in this study (female = 100, males = 177, median age = 61.81 years) Patients with all tumor stages (T1 = 38, T2 = 105, T3 = 102, T4 = 31, unknown = 1), nodal and metastasis status (N0 = 71 N+ = 206, M0 = 196, M1 = 81) were included. Data on lymphatic infiltration was available in 257 cases, data on venous infiltration in 274 cases. Lymphatic infiltration was observed in 165 patients (59.6%), venous infiltration in 90 patients (32.5%). The mean overall survival was 57.25 months (95% CI:
49.23–65.27), the mean disease-specific survival was 70.08 months (95% CI: 60.60–79.56).

**Activin protein expression is common in AEG/ASs and inversely correlated with tumor size, lymph node and distant metastasis**

High activin protein expression was observed in 72 out of 277 primary tumors (26.0%). We detected a mixed expression pattern of activin subunit inhibin beta A in tumor cells with predominantly cytoplasmic and marginally nuclear protein expression. Heterogeneity with regards to activin expression scores in between the two TMA cores per tumor sample was very low (difference > 1 point in either intensity or positive tumor cell subscores in 7 out of 277 cases corresponding to 2.4% of cases). Activin protein expression scores were negatively correlated with tumor size, lymph node and distant metastasis ($p < 0.041$, $p = 0.014$ and $p = 0.003$ respectively). Accordingly, we observed a statistically significant inverse correlation of activin expression with UICC stages (see Table 1).

Activin expression score was not significantly correlated with tumor grading ($p = 0.35$). With regard to the Lauren classification, activin expression scores did not differ significantly between tumors of the intestinal, diffuse or mixed type ($p = 0.11$). Higher activin protein expression scores correlated with tumors classified as expansive as opposed to infiltrative following the Ming Classification ($p = 0.034$).

**CD4+ T-helper cell tumor invasion correlates with higher activin expression**

As immune modulating effects of activin are reported in other tumor entities, we decided to investigate tumor T-cell infiltration as possible confounding factor. We observed high infiltration of T-cells positive for CD3+, CD4+ and CD8+ in 118, 98 and 120 of tumors respectively (42.6, 35.4, 43.3% of cases). When correlating T-cell infiltration with activin protein expression, we observed no statistically significant correlation of activin expression with CD3+ or CD8+ T-cells, but a statistically significant correlation with CD4+ T-helper-cells ($p < 0.009$). Additionally, we correlated activin with PD-1 and PD-L1 expression in lymphocytes and tumor cells. There was a trend towards higher activin protein expression in tumors with lymphocytes expressing PD-1 and PD-L1 respectively, but this trend did not reach statistical significance ($p = 0.051$ and $p = 0.065$ respectively).

**Activin protein expression is positively correlated with longer overall survival, especially in tumors with high CD4+ cell count**

Activin protein expression showed a statistically significant correlation with overall survival (see Fig. 2a) (72.1+/−8.27 versus 51.2+/−4.6 months, $p < 0.01$). The correlation of activin protein expression and tumor specific survival did not reach statistical significance. When investigating the correlation of activin and overall survival in tumors of different UICC stages, the effect was predominantly found in tumors of UICC stage III (35.5+/−9.2 versus 15.6+/−2.4 months, $p < 0.01$) (see Fig. 2b). In a multivariate analysis, only age and UICC stage remained statistically significantly correlated with overall survival (see Table 2).

As published before, CD4+ T-cell infiltration is correlated with longer survival in AEG/AS patients (Fig. 2C, [29]). Interestingly, when investigating the correlation of high activin protein expression and CD4+ T-cells, we observed improved survival solely in patients with tumors expressing both high activin levels and high CD4+ infiltration (median survival 86.8 months +/− 12.1). Tumors with either exclusively high CD4+ cells or exclusively high activin protein expression did not exhibit a longer survival (median survival 55.9+/−8.7 and 57.4+/−11 months) when compared with tumors low in CD4+ cell infiltration and activin expression (median survival 47.1+/−5.7 months) (Fig. 2d).

**Discussion**

This study presents data on the protein expression of the activin homodimer inhibin beta-A subunit in a comprehensive European cohort of AEG/AS tumors.

Our cohort studied encompasses 277 patients treated at a single center primarily by surgery, we observed high activin homodimer subunit inhibin beta A expression in roughly a quarter of the cases (71/277, 26.0%). Activin expression correlated inversely with tumor stage, and as therefore expected, we observed a statistically significant higher overall survival in patients with tumors exhibiting high activin expression (72.1+/−8.3 versus 51.2+/−4.6 months, $p < 0.01$). This effect was predominantly found in tumors of UICC stage III (35.5+/−9.2 versus 15.6+/−2.4 months, $p < 0.01$). Additionally, higher activin expression scores were seen in tumors of the expansive type rather than tumors of the infiltrative type following the Ming-Classification ($p = 0.04$). A multivariate regression did not show...
Fig. 2 (See legend on previous page.)
activin subunit protein expression as independent predictor of survival, which is most probably due to the strong inverse correlation of activin with tumor stage in the cohort studied.

These data are complementing prior in vivo data demonstrating a growth inhibiting effect of activin on gastric cancer cell lines [22, 23]. Studies investigating the mRNA expression of activin in AEG/AS cohorts reported a shorter overall survival [25, 27]. This discrepancy in findings could be explained by post transcriptional control of activin levels, as well as genetic differences in the cohorts, as the cohort studied herein is the first predominantly European cohort investigated for activin protein expression in AEG/ASs. Arguably tumor protein activin expression is a more accurate measurement of activin signal activation as compared to activin mRNA. In our cohort, the correlation between overall survival and activin subunit protein expression was most pronounced in UICC stage III cancers. This observation might be due to a stronger pro-proliferative effect through other pathways in these tumors when compared to UICC stage I and II cancers which is mitigated in cases of higher activating protein expression, but further studies are needed. The higher activin expression in tumors of the expansive type rather than tumors of the infiltrative type is most likely due to the correlation of both expansive type [31] as well as activin expression with lower tumor stage.

No correlation was found between activin expression in metastases or lymph nodes and overall survival, albeit a smaller cohort size reduces the power to detect a difference in these cases.

Interestingly, a correlation of activin protein expression and CD4+ T-helper cell infiltration was found, but not overall CD3+ T-cell infiltration or CD8+ cytotoxic T-cell infiltration.

We previously reported on longer survival of patients with AEG/ASs exhibiting high CD4+ cell infiltration [29]. Even though a non-statistical significant trend towards correlation between activin protein expression and overall survival was seen in tumors exhibiting a low CD4+ T-cell infiltration (high 57.4 +/- 11.0 months versus low 47.1 +/- 5.7 months, p = n.s.), the effect was more pronounced in tumors with high CD4+ T-cell infiltration score (88.8 +/- 12.1 months versus 55.9 +/- 8.6, p < 0.05). Interestingly, the longer overall survival from high CD4+ T-cell infiltration and high activin expression were reliant on each other. This finding strongly hints towards a tumor suppressive effect, activin exerts through a yet unspecified effect on CD4+ T-cells infiltrating the tumor. Whether this presumed effect is a direct effect on

| Clinical Factor | Univariate | Multiple Cox Regression |
|-----------------|------------|-------------------------|
|                 | Mean Survival | CI | p | HR | CI | p |
| **Total patients** | 57.79 | 49.7–65.886 | | | | |
| **Age** | | | | | | |
| ≤ 65 years | 64.66 | 53.39–75.92 | 0.028 | 1.409 | 1.039–1.911 | 0.027 |
| > 65 years | 47.39 | 36.86–57.88 | | | | |
| **Sex** | | | | | | |
| Female | 60.54 | 46.93–74.12 | 0.629 | 0.838 | 0.616–1.139 | 0.258 |
| Male | 54.34 | 44.74–63.94 | | | | |
| **UICC stage** | | | | | | |
| UICC I | 113.52 | 96.68–130.34 | <0.001 | 2.332 | 1.985–2.740 | <0.001 |
| UICC II | 73.05 | 57.85–88.26 | | | | |
| UICC III | 22.44 | 14.97–29.91 | | | | |
| UICC IV | 11.85 | 9.23–14.473 | | | | |
| **Ming** | | | | | | |
| Expansive | 63.55 | 51.51–75.69 | 0.033 | 1.122 | 0.814–1.547 | 0.482 |
| Infiltrative | 49.79 | 39.84–59.74 | | | | |
| **CD4** | | | | | | |
| High | 70.34 | 55.35–85.34 | 0.017 | | | |
| Low | 49.02 | 38.97–59.06 | | | | |
| **Activin** | | | | | | |
| High | 72.13 | 55.92–88.34 | 0.014 | | | |
| Low | 51.28 | 42.35–60.20 | | | | |
lymphocytes or an indirect effect through activins effect on tumor epithelium or fibroblasts and the induction of other signaling molecules cannot be said at this point. Due to the correlative nature of data presented here, it is also conceivable that combined high activin expression and CD4+ T-cell infiltration a marker for a yet to specify tumor subtype. CD4+ T-cells are a heterogeneous group of cells including pro- and anti-inflammatory T-helper cells (Th1 and Th2), IL-17 producing Th17 cells, and regulatory T-cells, and further subclassification of CD4+ T-cells should be performed in future studies to possible discern mechanistic interaction between activin and CD4+ T-cells in AEG/AS tumors, especially given the recent data on activin driving differentiation towards Th17 cells [21].

Several limitations should be kept in mind when interpreting the data presented here. TMAs are a valuable tool to assay protein expression in large cohorts such as ours, but some concern exists due to the incomplete reflection of tumor heterogeneity, especially with regards to tumor infiltrating lymphocytes. The heterogeneity in between TMA cores, at least for activin staining was low (difference >1 point in either intensity or positive tumor cell sub-scores in 2.4% of cases), and the tumor infiltrating lymphocyte working group sees TMAs as adequate tool for rapid evaluation of large clinical cohorts [32]. Nevertheless, full reflection of tumor heterogeneity was not possible due to our approach, a limitation that should be kept in mind. In our study, we investigated protein expression of the activin homodimer subunit inhibin beta A. Inhibin beta A dimerizes with either the inhibin alpha subunit or Inhibin beta subunit, for which several isoforms exist. A cross-reactivity with other homo- and heterodimers of inhibin beta A besides activin A cannot be ruled out by immunohistochemistry, an important limitation when interpreting the results of this study.

In conclusion, the data presented herein show a correlation of higher activin tumor protein expression and longer overall survival in a cohort of patients with AEG/AS tumors. The data imply a net anti-tumorigenic effect of activin signaling in this tumor entity, albeit due to the context specific nature of activin signaling observed in other tumor entities a more distinguished function with a net pro tumorigenic effect in a subset of tumors is conceivable. Therefore, further mechanistic in vitro and in vivo studies of activin in the context of AEG/ASs, especially with regards to its effect on CD4+ tumor infiltrating lymphocytes, are warranted.

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**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12885-022-10016-5.

**Additional file 1:** Supplemental Table S1. Overview of visualized antigens and visualizing agent.

**Additional file 2:** Figure S1. Distribution of activin high and activin low expressing tumors by tumor stage illustrating the inverse correlation of activin with UICC tumor stage.

**Authors’ contributions**

Study was designed by JJS and CT. Cohort was designed and created by BR, US and CT. Histology was assessed by WM and EB. Staining was performed by AAK, MP and EB. Scoring of staining was done by JJS and CT. Manuscript was drafted by JJS and revised by SD and CT. Data interpretation and statistical analysis was done by JJS, SD and CT. Confirmation of staining pattern and scoring for activin subunit inhibin beta A was performed by AA. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Declarations**

**Ethics approval and consent to participate**

This study has been approved by the by the Institutional Review Board of the Charité – Universitätsmedizin Berlin (EA4/115/10) and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All patients included in this study gave their informed consent prior to their inclusion.

**Consent for publication**

No identifiable individual person’s data is included in this manuscript.

**Competing interests**

The authors declare that they have no conflicts of interest.

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