

# Circulating levels of TNF-related apoptosis inducing-ligand are decreased in patients with large adult-type granulosa cell tumors—implications for therapeutic potential

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**Abstract** Targeted treatments are needed for advanced adult-type granulosa cell tumors (AGCTs). We set out to assess tumor tissue and circulating levels of TNF-related apoptosis-inducing ligand (TRAIL), a promising anti-cancer cytokine, in patients affected by AGCT. We analyzed tissue expression of TRAIL in 127 AGCTs using immunohistochemistry or RT-PCR. Soluble TRAIL was measured by means of ELISA from 141 AGCT patient serum samples, as well as the conditioned media of 15 AGCT patient-derived primary cell cultures, and the KGN cell line. Tissue and serum TRAIL levels were analyzed in relationship with clinical parameters, and serum estradiol, FSH, and LH levels. We found that AGCT samples expressed TRAIL mRNA and protein at levels comparable to normal granulosa cells. AGCT cells did not release soluble TRAIL. TRAIL protein levels were decreased in tumors over

10 cm in diameter ( $p=0.04$ ). Consistently, circulating TRAIL levels correlated negatively to tumor dimension ( $p=0.01$ ). Circulating TRAIL levels negatively associated with serum estradiol levels. In multiple regression analysis, tumor size was an independent factor contributing to the decreased levels of soluble TRAIL in AGCT patients. AGCTs associate with significantly decreased tumor tissue and serum TRAIL levels in patients with a large tumor mass. These findings encourage further study of agonistic TRAIL treatments in patients with advanced or recurrent AGCT.

**Keywords** Estradiol · FSH · GATA4 · Granulosa cell tumor · LH · Ovarian cancer · Targeted treatment · TRAIL

## Background

Ovarian granulosa cell tumors (GCTs) are hormonally active sex cord-stromal tumors, which represent 5 % of all ovarian malignancies. Adult-type GCTs (AGCTs) are usually diagnosed at an early stage, but recurrences occur in 20–30 % of the patients after a median time of 4–7 years [1, 2]. These recurrences are associated with a high mortality, and early detection and complete tumor removal are key elements in the treatment of recurrent AGCT [3, 4]. Adjuvant treatments have limited proven efficacy on recurrent or advanced-stage AGCT [5], and biologically targeted treatment modalities are lacking.

Clinical trials show a promising activity of the TNF-related apoptosis-inducing ligand (TRAIL) cytokine and of agonistic anti-TRAIL receptor antibody against a variety of solid tumors [6–11]. TRAIL is expressed by different cell types belonging predominantly to the immune system [12] either as type II transmembrane homo-trimer or as a soluble protein, detectable in circulation under physiological conditions [12]. The best

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characterized role of TRAIL is to act as an anti-cancer molecule [13, 14]; this is also clearly demonstrated in TRAIL knockout animal studies showing that lack of TRAIL promotes chronic inflammation, tumorigenesis, and metastasis [15, 16]. We and others have previously demonstrated that adult ovaries expressed TRAIL and its receptors TRAIL-R2, TRAIL-R3, and TRAIL-R4 in granulosa cells of small and larger antral follicles [17, 18]. Moreover, TRAIL pathway components are active in AGCTs, and recombinant human TRAIL induces apoptosis in AGCT cells in vitro [17–21]. In addition, clinical response of 23 % reduction in measurable disease was observed in a patient with advanced AGCT, treated with 4 mg/kg of an agonistic anti-TRAIL-receptor-2 antibody [11].

AGCT has a distinct molecular background; most AGCTs harbor a single somatic point mutation in a gene encoding for transcription factor FOXL2 [22, 23]. Still, the pathogenesis of AGCTs remains largely unraveled. Originating from the hormonally active granulosa cells, AGCTs produce, e.g., estradiol, anti-Müllerian hormone, and inhibins, and express receptors for follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (reviewed in [24]). Recently, estradiol was found to negatively regulate TRAIL expression in leukocytes [25].

On these bases, we evaluated the tumor tissue expression and the circulating levels of TRAIL in patients affected by AGCT, and evaluated the levels in relation to clinical and hormonal parameters.

## Patients and methods

### Real-time PCR and primary cell cultures

Total RNA was extracted from 46 primary tumor samples, and one human granulosa-luteal cell sample, three primary AGCT cell samples, and the KGN cell line and using the Qiagen RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the supplier's instructions, and as previously described [26]. Once verified the quality of RNA preparation by agarose gel, total RNA was transcribed into cDNA, using the QuantiTect® Reverse Transcription kit (Qiagen). *TRAIL* gene expression was analyzed using the SYBR Green-based real-time quantitative polymerase chain reaction (RT qPCR) detection method with SA Biosciences RT<sup>2</sup> Real-Time™ Gene expression assays, which include specific validated primer sets and PCR master mix (SABiosciences, Frederick, MD). The samples were run in triplicate using the real time thermal analyzer Rotor-Gene™ 6000 (Corbett, Cambridge, UK), or with ABI Prism 7900HT (Thermo Fisher Scientific, MA, USA) as previously described [21, 26]. Expression values were normalized to the housekeeping gene *POLR2A* or *Cyclophilin* amplified in the same sample and expressed in arbitrary units as described [27].

During 2007–2010, we obtained ten primary and five recurrent AGCTs for primary cell cultures, which were established as

described [21]. In brief, the fresh tumor tissue was mechanically minced and treated with 0.5 % collagenase (Sigma-Aldrich Corp., St. Louis, MO), filtered through a 140-µm filter mesh, and the single cells were plated in the DMEM/F12 containing 10 % fetal bovine serum. The cells were cultured in 5 % CO<sub>2</sub> at +37 °C temperature. All the primary AGCT cells and the KGN cells harbored the c.402C->G (C134W) mutation in FOXL2 [22]. KGN cells were cultured as described [28]. Cell culture supernatant was collected after 2–5 days depending on cell confluence and analyzed for the release of soluble TRAIL by ELISA (R&D Systems), according to instructions and as previously described [13].

### Immunofluorescence

Primary AGCT cells were plated on 4-well chamber glasses on day 6 after primary culture establishment. On day 10, the cells were fixed with 4 % PFA, permeabilized with 0.1 % PBS-Triton-X, blocked with 1 % BSA, and stained with primary antibodies for FOXL2 (sc-25825, Santa-Cruz Biotechnologies, TX, USA) and TRAIL (sc-1891, Santa-Cruz) in 1:100 dilution at +4 °C o/n. Secondary antibody only was utilized as a control (data not shown). Secondary antibody in 1:500 dilution with Alexa fluor 488 (GFP) conjugate was incubated for 1 h at room temperature, and the nuclei were counterstained with DAPI.

### Immunohistochemistry

An AGCT tissue microarray of quadruple core samples from 71 primary and 12 recurrent AGCT was used for expression analysis; three normal ovarian tissue samples were used for controls. Clinicopathologic data of the patients and samples in the TMA are presented in Table 1. The AGCT diagnoses were verified by FOXL2 mutation and pathological review. Immunohistochemical staining was performed as described [17]. The primary antibody anti-human TRAIL (sc-1891, Santa-Cruz) was used in a 1:50 dilution. Secondary antibody only served as a negative control. The stained tissue sections were examined using a Leica DM RXA microscope (Leica microsystems GmbH, Wetzlar, Germany) ×20 and ×40 objectives, and images were acquired using an Olympus DP70 camera and DP-capture software (Olympus, Center Valley, PA, USA). Researchers (AF and UMH) independently analyzed the intensity of staining from four cores of each tumor in relation to granulosa cells in the normal ovary. The expression levels were correlated to clinical data as described previously [1].

### Patients and serum samples

We collected 141 serial serum samples from 83 AGCT patients treated at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital during 2007–2012. The

**Table 1** Clinicopathologic data of the patients (A) and samples (B) in the TMA

A Patients <i>N</i> = 79	<i>N</i> (% of total)	
Age at diagnosis, years <sup>a</sup>	50 (26–81)	
Tumor stage at diagnosis		
I	68 (86)	
II	8 (10)	
III	3 (4)	
Initial treatment		
Surgery only	62 (79)	
Surg + Ch	12 (15)	
Surg + Rad	2 (2)	
Surg + Ch + Rad	3 (4)	
Follow up time, years <sup>a</sup>	15.3 (0.7–38.6)	
Recurrence		
Yes	27 (34)	
No	52 (66)	
Survival		
Alive	45 (57)	
Dead of AGCT	19 (24)	
Dead of other	15 (19)	
<i>B</i> Tumor characteristics	<i>n</i> (% of total)	
Total <i>n</i> = 81	Primary	69 (86)
	Recurrent	12 (14)
Subtype	Sarcomatose	31 (38)
	Differentiated	50 (62)
	Nuclear atypia	High 19 (23)
		Low 62 (77)
	Mitotic Index	High 21 (26)
		Low 60 (74)
	Tumor size	≥10 cm 31 (38)
		<10 cm 50 (62)

*N* number of patients, *n* number of samples, *Surg* surgery, *Ch* chemotherapy, *Rad* radiotherapy

<sup>a</sup>Median (range)

**Table 2** Clinicopathologic data of the patients (A) and samples (B) in the serum analysis

A Patients <i>N</i> = 83	<i>N</i> (% of total)
Age at diagnosis, years <sup>a</sup>	52 (19–81)
Tumor stage at diagnosis	
I	77 (93)
II	5 (6)
III	1 (1)
Initial treatment	
Surgery only	71 (86)
Surg + Ch	12 (14)
Follow up time, years <sup>a</sup>	8.4 (0.7–49.6)
Recurrence	
Yes	21 (25)
No	62 (75)
Survival	
Alive	81 (98)
Dead of AGCT	2 (2)
<i>B</i> Serum Samples	Total <i>N/n</i>
Total	141/83
WD	39/30
Primary	17/17
Stage Ia	11/11
Stage Ic	6/6
Recurrent	22/13 <sup>b</sup>
DF	102/77 <sup>b</sup>
PreMP <sup>c</sup>	18 (13)
PostMP <sup>c</sup>	123 (87)
Tumor size <sup>a</sup>	7.0 (2.0–30.0)

*n* number of samples, *N* number of patients, *Surg* surgery, *Ch* chemotherapy, *WD* With Disease, *DF* Disease-free, *MP* Menopausal

<sup>a</sup>Median (range)

<sup>b</sup>Serial samples from multiple recurrences

<sup>c</sup>MP status at sample retrieval; “premenopausal” (PreMP) if the patient had and one or two ovaries, and menopause was not indicated in the medical records, and “postmenopausal” (PostMP) if the both ovaries had been removed, or the patient was postmenopausal according to medical history

ethical committee of Helsinki University Central Hospital (HUCH) and the National Supervisory Authority for Welfare and Health in Finland approved the study, and all patients gave their informed consent. The patient and sample details are displayed in Table 2. The “with disease” (WD) samples were drawn within a month before AGCT treatment, and the “disease-free” (DF) samples were drawn after a minimum of 3 months from the treatment, at the time of a clinical control visit when the patient was disease-free. The samples were classified according to patient’s menopausal status at sample retrieval; “premenopausal” (PreMP) if the patient had one or two ovaries, and menopause was not indicated in the medical records (e.g., cessation of regular bleedings, presence of

menopausal symptoms, use of hormonal replacement therapy), and “postmenopausal” (PostMP) if both ovaries had been removed independent of age, or the patient was postmenopausal according to medical history. The median age was 41 years (range 26–54 years) for PreMP patients and 60 years (range 44–86 years) for postMP patients.

Serum samples were prepared and stored at –80 °C until analyzed for TRAIL with ELISA (R&D Systems, Minneapolis, MN, USA) according to instructions and as previously described [13, 29]. Sensitivity of the assay was 2.86 pg/ml, with the intra- and inter-assay variabilities of 3.9 and 6.0 %, respectively. Blood leukocyte counts and serum levels of estradiol (nmol/L), FSH, LH (IU/L) were measured in the hospital routine with

fluoroimmunoassays (AutoDELFIA™, Wallac, Turku, Finland). For analysis of continuous data, the values below detection limit <0.1 (nmol/L for estradiol, IU/L for FSH and LH) were set to 0.05 (nmol/L or IU/L).

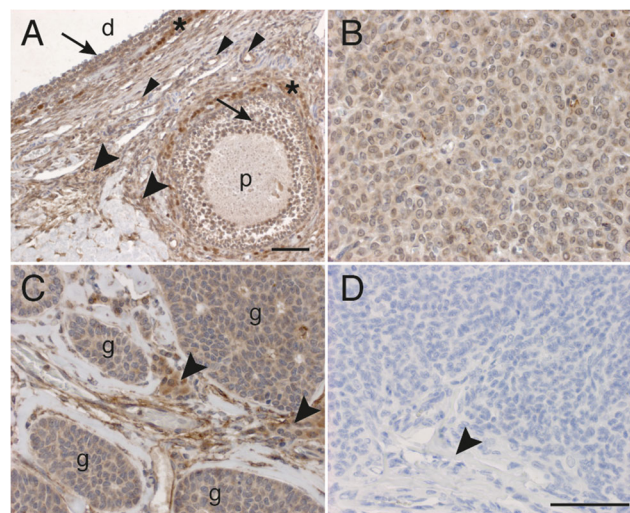
### Statistical analysis

TRAIL protein expression levels were correlated to clinical parameters with contingency tabling and Fisher's exact test. The serum TRAIL data were tested to follow normal distribution with Shapiro-Wilk's test and serial measurements were analyzed with generalized linear model. Serum FSH, LH, and estradiol levels and tumor size differed from normal distribution even after performing logarithmic transformation and thus Mann–Whitney *U* test and Spearman's rho were utilized. For multiple regression, a generalized linear model and maximum likelihood ratio estimation were utilized for tumor size as a continuous variable (with the most correlations in univariate analysis), menopausal status, estradiol (nmol/L), and FSH and LH (IU/L) levels, with soluble TRAIL (pg/ml) as the dependent variable.  $p < 0.05$  was considered significant. The analyses were performed with JMP version 7.0.1 (SAS Institute Inc., Cary, NC) software.

## Results

### TRAIL protein expression is reduced in large GCTs

We have previously shown that the TRAIL pathway plays an essential role in granulosa cells [17]. To investigate the role of TRAIL in malignant granulosa cells, we now analyzed the expression of TRAIL in AGCTs at both messenger RNA (mRNA) and protein levels. In an analysis of 46 primary tumor tissues, TRAIL mRNA was expressed at variable amounts, with mean  $1.6 \pm 1.2$  arbitrary units, as compared to 0.08 arbitrary units in normal granulosa-luteal cells (Supplemental Figure 1, Supplemental Table 1). We found no correlations of TRAIL mRNA levels to tumor size or other clinicopathological characteristics (data not shown). In immunohistochemical analysis of normal human ovary, TRAIL protein was expressed in theca and granulosa cells, as well as in the ovarian stroma (Fig. 1a). In AGCTs, TRAIL protein was expressed in 48 (59 %) of the tumors at a level comparable to a normal granulosa cell, staining mostly diffusely in the tumor cells (Fig. 1b) and more strongly in the tumor stroma (Fig. 1c). Thirty (37 %) AGCTs exhibited low-intensity staining, and three (4 %) remained entirely negative for TRAIL protein. Upon correlations between clinical and immunohistochemical data, TRAIL protein expression was significantly lower in tumors over 10 cm in diameter when compared to smaller tumors ( $p = 0.04$ , OR 0.4 95% CI 0.2–0.97). TRAIL protein expression level was similar in primary ( $n = 71$ ) and recurrent ( $n = 12$ ) tumor samples, and the



**Fig. 1** Immunohistochemistry demonstrating TRAIL protein expression in AGCTs. **a** TRAIL staining in a large dominant follicle (*d*) and a primary follicle (*p*) of a normal human ovary. Positive staining is visible in granulosa cells (*arrows*), thecal cells (*asterisks*), in endothelial cells (*small arrowheads*), and in the stroma (*big arrowheads*). **b** AGCT of the sarcomatose subtype showing diffuse TRAIL staining. **c** AGCT with TRAIL staining in the tumor islets (*g*) and in the tumor stroma (*arrowheads*). **d** Secondary antibody only. Tumor cells and tumor stroma (*arrowhead*) are negative. Original magnification  $\times 20$  in **a**,  $\times 40$  in **b–d**, scale bars 20  $\mu\text{m}$

expression levels did not correlate to tumor subtype, mitotic index, nuclear atypia, tumor stage, or recurrence probability (data not shown). TRAIL expression correlated positively to the expression levels of the TRAIL receptors DR4 ( $p = 0.006$ , OR 11.3, 95% CI 1.4–91.9) and DR5 ( $p = 0.02$ , OR 8.9, 95% CI 1.1–73.1) previously analyzed in [21]. However, DR4 and DR5 expressions did not correlate to tumor size (data not shown). To further understand the reason for decreased TRAIL protein expression in large AGCTs, we correlated the TRAIL levels to factors linked to granulosa cell proliferation and apoptosis, such as GATA4, Bcl2, and VEGF previously analyzed in [21, 30–32]. Interestingly, we found that GATA4 and TRAIL levels positively correlated in the tumor tissue (OR 4.4, 95% CI 1.7–11.6,  $p = 0.003$ ). However, TRAIL expression did not correlate with the expression of VEGF, VEGF receptors, tumor microvessel density, or Bcl2 expression (data not shown).

To investigate whether AGCT cells secrete soluble TRAIL, we next analyzed TRAIL levels from the cell culture supernatants collected from 15 AGCT primary cell and KGN cell cultures. We found that AGCT cells did not secrete measurable amounts of soluble TRAIL into the supernatant (undetectable, data not shown). However, TRAIL mRNA was expressed in three primary AGCT cell preparations (mean  $= 32.9 \pm 25.5$  arbitrary units), at levels comparable to data previously obtained from normal endothelial cells (mean  $= 35.9 \pm 36$  arbitrary units), but at lower levels than that in peripheral blood mononuclear cells (mean  $= 63.4 \pm 48.7$  arbitrary units) [25]. When studied

with immunofluorescence staining, the primary tumor cells expressed FOXL2 protein as a marker for AGCT origin (Fig. 2a–c) but remained negative for TRAIL expression (Fig. 2d–h).

### Circulating TRAIL levels are decreased in patients with large AGCTs

To evaluate the dynamics of circulating TRAIL in AGCT patients, we next analyzed serum TRAIL concentrations from 141 serum samples, before (WD) and after (DF) tumor treatment. We found that the tumor itself had little effect on circulating TRAIL levels, as there was no significant difference in WD compared to DF samples, (data not shown). However, soluble TRAIL levels were significantly lower in patients with larger tumors of over 10 cm in diameter (mean 83.6, SD 29.2, pg/ml), than in patients with tumors less than 10 cm in diameter (mean 122.1, SD 30.2 pg/ml;  $p=0.002$ ) (Fig. 3a). Consistently, soluble TRAIL levels inversely correlated to the tumor diameter as a continuous variable ( $r=-0.4$ ,  $p=0.01$ ) (Fig. 3b). Upon correlations to other clinical parameters, we found that TRAIL levels did not correlate to the patient age at sample retrieval, body weight or height, or blood leukocyte counts (data not shown).

### Tumor size independently correlates to circulating TRAIL levels

Circulating TRAIL levels have been shown to correlate inversely with patient's estradiol levels [33], and we next analyzed whether soluble TRAIL levels seen in large AGCTs associate with the patient's menopausal status, estradiol, and FSH and LH levels. In patients with AGCT (WD), we found that circulating TRAIL levels negatively associated with serum estradiol levels (Spearman's Rho  $-0.32$ ,  $p=0.05$ ), whereas no

correlations were found to menopausal status, FSH, or LH levels (data not shown).

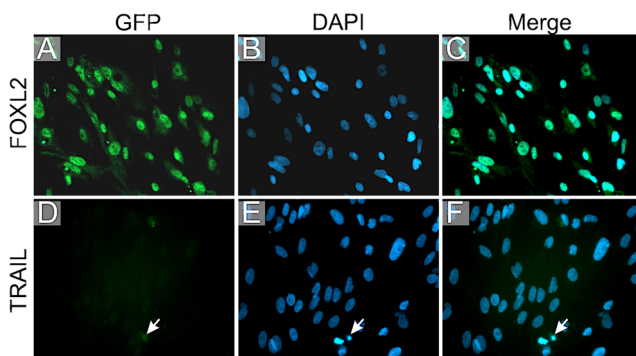
We noted that larger tumors associated with PreMP status, higher estradiol, and lower serum FSH and LH levels (Table 3a). Menopausal status, estradiol, FSH and LH levels correlated to each other, as expected (data not shown). In order to verify which factor independently contributed to the decreased TRAIL levels in AGCT patients, we next performed a multiple regression analysis for tumor size, menopausal status, and estradiol, FSH, and LH levels with serum TRAIL as the dependent variable (Table 3b). The analysis indicated that tumor size was the only independent factor influencing circulating TRAIL levels in AGCT patients ( $p=0.007$ ).

### Discussion

Despite improvements in the treatment modalities over the past decades, ovarian cancer remains the leading cause of death from gynecological cancers. AGCTs generally show an indolent course of disease; however, recurrences occur in 30 % of the patients and the majority of these patients will die of their disease [1, 34, 35]. Given the fact that current chemotherapeutics have only limited effect on AGCTs [5, 36], new, targeted treatments are critically needed for patients with advanced AGCT.

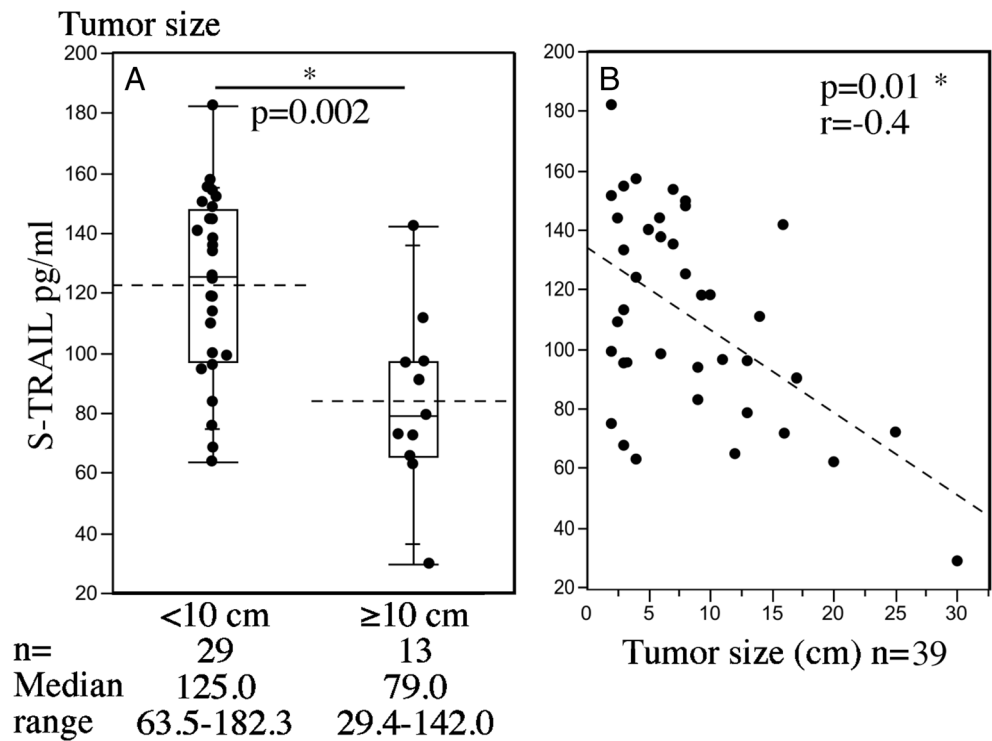
TRAIL is a pro-apoptotic factor with promising anti-cancer activity in a multitude of solid tumors [6–11]. Previous studies have documented the expression of TRAIL in granulosa cells [17] and TRAIL receptors in normal and malignant granulosa cells [21], suggesting a role for the TRAIL/TRAIL receptor system in regulating normal and malignant granulosa cell apoptosis. Recombinant human TRAIL (rhTRAIL) induces apoptosis in AGCT cells in vitro [17, 21]. Moreover, rhTRAIL was shown to induce the activation of Caspase 3/7 and subsequent cell death with lower concentrations in the primary AGCT tumor cells when compared to the KGN cell line [21]. Preliminary clinical data also suggests that AGCTs are susceptible to agonistic TRAIL therapy also in human AGCT patients [11].

We herein find that AGCTs generally express TRAIL protein at levels comparable to normal granulosa cells. However, tissue expression levels were decreased in large AGCTs. TRAIL protein expression levels positively correlated with those of transcription factor GATA4, which regulates apoptosis in AGCTs [21], and has been shown to be an important delineator of AGCT prognosis [1, 30]. Moreover, GATA4 has been shown to protect AGCT cells from TRAIL-induced apoptosis in vitro [21]. Therefore, high expression of GATA4 in AGCTs can be speculated to lead to resistance of TRAIL-mediated apoptosis, and contributing to increased tumor growth. However, we could not detect TRAIL protein in cultured primary AGCT cells. Moreover, in immunohistochemistry, TRAIL protein expression was also detected in the tumor stroma, and



**Fig. 2** Immunofluorescent staining of primary cultured AGCT cells. First column; antigen staining visualized with GFP in *green*, second column; DAPI showing the nuclei in *blue*, third column; merge. **a–c** FOXL2 protein as a marker for AGCT cells localizes primarily in the nuclei of tumor cells. **d–f** AGCT cells are predominantly negative for TRAIL protein expression. A small amount of TRAIL staining is seen around an apoptotic AGCT cell (*arrow*)

**Fig. 3** Serum TRAIL levels are decreased in patients with large AGCTs and correlate negatively with tumor size. **a** S-TRAIL levels in tumors less than 10 cm and in tumors equal to or more than 10 cm in diameter. **b** S-TRAIL levels in correlation with tumor size as the largest diameter. *r* Spearman’s rho. An asterisk indicates a significant difference with *p* < 0.05



TRAIL mRNA was present at high levels in the primary AGCT tumor samples, consisting also of tumor stroma. These findings suggest that TRAIL protein is primarily produced by the tumor

stroma and/or infiltrating blood/immune cells. Thus, it can be speculated that the decreased TRAIL production in the stroma of large tumors contribute to AGCT progression via decreasing

**Table 3** Tumor size independently correlates to serum TRAIL levels. (A) Univariate analyses to tumor size. (B) Multiple regression with serum TRAIL as the dependent variable

	Tumor size (cm)	(continuous) <i>p</i> value	Tumor size <10 cm	(categorical) ≥10 cm	<i>p</i> value
<b>A</b> Correlatons to tumor size as continuous and categorical value					
PreMP	mean ± SD; 14.7 ± 11.0		<i>n</i> = 3	<i>n</i> = 3	
PostMP	mean ± SD; 7.5 ± 5.0	0.01 <sup>a</sup>	<i>n</i> = 25	<i>n</i> = 8	NS <sup>b</sup>
Estradiol nmol/L	<i>r</i> = 0.41	0.01*	median 0.07 range 0.01–0.33	median 0.21 range 0.04–0.93	NS <sup>c</sup>
FSH IU/L	<i>r</i> = -0.64	<0.0001*	median 15.3 range 0.05–101	median 1.2 range 0.05–50.1	0.006 <sup>bc</sup>
LH IU/L	<i>r</i> = -0.74	<0.0001*	median 23.5 range 3–66.9	median 5.9 range 0.05–23.3	0.003 <sup>bc</sup>
<b>B</b> Multiple regression of serum TRAIL as the dependent variable					
	Estimate	95%CI	<i>p</i> value		
Intercept	138.4	101.5–175.3	<0.0001*		
Tumor size	-3.0	-5.4–-0.7	0.001*		
PreMP	-15.7	-35.5–4.2	0.1		
Estradiol	-23.0	-80.5–34.6	0.4		
FSH	0.4	-0.2–1.0	1.0		
LH	-0.9	-1.9–0.2	0.1		

PreMP premenopausal, Postmp postmenopausal, SD standard deviation, CI confidence interval, IU international units, L liter, NS not significant

*r* = Spearman’s rho, \*indicates *p* < 0.05

<sup>a</sup> One-way ANOVA

<sup>b</sup> Fisher’s exact test

<sup>c</sup> Mann-Whitney *U* test

apoptosis in the tumor cells expressing the TRAIL receptors DR4 and DR5 [21] and, potentially, also via impairing tumor defense mechanisms [37–39].

Circulating TRAIL levels were similar in patients with or without AGCT; however, the levels were significantly decreased in AGCT patients with a large tumor mass, consistent with our findings in the tumor tissue. AGCTs are known to produce and release estrogens [24], and recent in vitro data showed that estradiol downregulates the production of TRAIL from leukocytes [25]. We found evidence that serum estradiol levels correlated positively to tumor size, and also negatively to circulating TRAIL levels in patients with AGCT. However, in multivariable analysis, tumor size independently associated with TRAIL levels, and therefore, it can be speculated that other factors, such as the tumor stroma, also contribute to the levels of circulating TRAIL. When designing optimal treatment strategies for advanced AGCTs, the targeted treatment preferably antagonizes with inherent pathogenetic factors, such as GATA4, and further studies are warranted to evaluate whether TRAIL agonists have such potential.

Utilizing a unique series of tumor tissue, cell culture, and serum samples, we showed that large AGCTs are associated with significantly decreased levels of tissue and serum TRAIL. Our results support the role of TRAIL in AGCT tumor progression, and encourage future studies on agonistic TRAIL treatments for advanced or recurrent AGCT.

*AGCT* adult-type granulosa cell tumor, *DF* disease-free, *FSH* follicle-stimulating hormone, *LH* luteinizing hormone, *PostMP* postmenopausal, *PreMP* premenopausal, *TMA* tumor tissue microarray, *TRAIL* TNF-related apoptosis-inducing ligand, *rhTRAIL* recombinant human TRAIL, *WD* with disease

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**Author contributions** AF collected the samples, produced and analyzed the data, carried out the statistical analyses and wrote the manuscript; GZ produced and analyzed the data and drafted the manuscript; U-MH and MP performed immunohistochemistry, cell culture studies, qPCR, and analyzed the data; PS, LU-K and MH collected the samples, conceived of the study and interpreted the data; All authors wrote the manuscript, and read and approved this version of the manuscript.

**Compliance with ethical standards** The ethical committee of Helsinki University Central Hospital (HUCH) and the National Supervisory Authority for Welfare and Health in Finland approved the study, and all patients gave their informed consent.

**Conflicts of Interest** None

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