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Combined STING levels and CD103+ T cell infiltration have significant prognostic implications for patients with cervical cancer

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ABSTRACT

Activation of STimulator of INterferon Genes (STING) is important for induction of anti-tumor immunity. A dysfunctional STING pathway is observed in multiple cancer types and associates with poor prognosis and inferior response to immunotherapy. However, the association between STING and prognosis in virally induced cancers such as HPV-positive cervical cancer remains unknown. Here, we investigated the prognostic value of STING protein levels in cervical cancer using tumor tissue microarrays of two patient groups, primarily treated with surgery (n = 251) or radio(chemo)therapy (n = 255). We also studied CD103, an integrin that marks tumor-reactive cytotoxic T cells that reside in tumor epithelium and that is reported to associate with improved prognosis. Notably, we found that a high level of STING protein was an independent prognostic factor for improved survival in both the surgery and radio(chemo)therapy group. High infiltration of CD103+ T cells was associated with improved survival in the radio(chemo)therapy group. The combination of STING levels and CD103+ T cell infiltration is strongly associated with improved prognosis. We conclude that combining the prognostic values of STING and CD103 may improve the risk stratification of cervical cancer patients, independent from established clinical prognostic parameters.

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Introduction

Cervical cancer is the most common gynecological cancer worldwide and the fourth leading cause of cancer-related death in women. The development of cervical cancer is largely caused by persistent infection with human papilloma virus (HPV), with type 16 and 18 accounting for approximately 70% of all cervical cancers.[1,2] Some studies have estimated the overall prevalence of HPV in cervical cancer as high as 99%[3], demonstrating that persistent infection with HPV is pivotal in the development of cervical cancer. However, not all HPV infections result in malignant transformation. The immune system is usually effective in eradicating the virus as a large majority of 90% of infections is cleared within 2 years[4]. Apart from preventing carcinogenesis by eradicating the HPV infection, the immune system also plays a protective role in early/developing malignancies by recognizing and destroying transformed cells and hence functioning as an important defense against cancer.[5] Recent evidence shows that during infection or cancer, STimulator of INterferon type 1 Genes (STING; also known as Transmembrane Protein 173 (encoded by *STING1*) is critical for activation of innate immunity. STING acts after binding to cyclic dinucleotides, which can be derived exogenously from bacteria or can be generated upon detection of foreign (e.g. viral) or host nucleic acids in the cytosol by Cyclic-GMP-AMP (cGAMP) Synthase (cGAS). STING ultimately controls the transcription of numerous host defense genes, including type 1 interferons (IFNs) and several cytokines via downstream signaling, including phosphorylation of interferon regulatory factor 3 (IRF3). Production of IFNs is essential for further activation of both the innate and adaptive immune response.[6] Recent studies using tumor mouse models underline the pivotal role of STING in anti-tumor immunity. Several of these studies showed that STING-deficient mice were more susceptible to developing cancer. These mice show an inadequate anti-tumor immune response and lost control of tumor growth.[7–10] Vice versa, high STING expression led to higher levels of IFN production and numbers of CD8+ tumor-infiltrating lymphocytes (TILs).[11,12] Furthermore, STING plays an essential role in dendritic cell recognition of dying tumor cells and priming of anti-tumor cytotoxic T cell (CTL) responses.[8,11,13] Loss or decreased expression of STING associates with poor prognosis in several human cancer types such as gastric cancer, hepatocellular carcinoma, head and neck squamous cell carcinoma (HNSCC), breast cancer and colorectal cancer.[14–19] Previously, STING protein expression was evaluated in high grade cervical intraepithelial neoplasia (CIN3).[20] Here, we evaluated STING protein expression and sought to investigate the prognostic value of STING in established cervical cancer. Moreover, we investigated how the prognostic value of STING relates to the prognostic value of CD103, an integrin that marks anti-tumor tissue-resident memory T cells and has

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high prognostic value in multiple cancer types, including cervical cancer.[21–24] We included two cervical cancer patient groups and studied pre-treatment STING and CD103 protein levels in relation to clinical characteristics. The first group consisted of patients primarily treated with surgery, the first choice of treatment in early stage of disease. The second group consisted of patients that received (chemo)radiation as the first modality of treatment, which is given as a first choice of treatment to patients with locally advanced cervical cancer. Additionally, we investigated the association between *STING1* (encoding STING) mRNA and survival in cervical cancer patients using RNA sequencing data from The Cancer Genome Atlas (TCGA) cervical carcinoma datasets.

Methods & materials

Patient selection

In the University Medical Center Groningen (UMCG) clinicopathologic and follow-up data are prospectively obtained during standard treatment and follow-up and stored in a computerized registration database. For the present study, cervical cancer patients (n = 679) treated between 1986 and 2004 in the UMCG were included in a separate anonymous database, in which patient identity was protected by study-specific, unique patient codes. Codes were only known by two dedicated data managers. Patients were treated with surgery or radio(chemo)therapy (RT/RCT), according to standard-of-care at time of treatment. Tumors were graded and classified by a pathologist specialized in gynecologic malignancies. Tumors were staged according to the Federation of Gynecology and Obstetrics (FIGO) guidelines.[25,26] Patients were divided in two groups based on their primary treatment; surgery or RT/RCT. Surgical treatment consisted of a radical hysterectomy combined with pelvic lymph node dissection. All patients that received RT or RCT as first modality of treatment were included in the RT/RCT group (see characteristics in Table 1). No significant differences in STING levels (including medians) and disease-specific survival (DSS) were observed between RT and RCT (Supplemental figure S1). Patients with disseminated disease were not excluded from analyses. The tissue used for the analyses was obtained from pretreatment biopsies. Using the registration database, all tissue specimens were identified by unique patient numbers and retrieved from the archives of the Department of Pathology. Therefore, according to Dutch law, no further Institutional Review Board approval was needed.

Tissue micro array construction

Material from the patients that met the inclusion criteria was used to construct a tissue micro array (TMA). The TMA was constructed by the pathology department of the UMCG as described previously.[27] One millimeter-sized tumor (center of the tumor) and tumor/stroma (invasive margin) cores of each tumor block were randomly distributed on the TMA in triplicate.

Immunohistochemical analysis of p16, STING and CD103 in cervical carcinoma

TMA slides were stained for p16 as surrogate marker for HPV status. Pretreatment of the TMA slides was performed using ULTRA Cell Conditioning 1 (Roche Ventana, Tucson, AZ, USA). Next, slides were incubated with ready-to-use primary anti-p16 mouse monoclonal antibody, clone E6H4, (Roche Ventana, Tucson, AZ, USA). After incubation, the primary antibody was visualized using the Optiview detection kit (Roche Ventana, Tucson, AZ, USA). The complete staining protocol was performed using the Ventana Benchmark ULTRA instrument (Roche Ventana, Tucson, AZ, USA), according to the instructions of the manufacturer. Appropriate washings were performed in between steps. Upon staining, slides were scanned using a NanoZoomer 2.0-HT multi slide scanner (Hamamatsu Photonics). Cores were independently scored by two researchers and a pathologist, blinded for clinicopathological data. A patient was annotated to be HPV-positive in case at least one tumor core was found to be p16 positive (Supplemental Figure S2).

Immunohistochemical analysis of CD103 and CD8 was performed previously.[23] To assess STING, TMA slides were first dewaxed in xylene and rehydrated using degrading concentrations of ethanol to distilled water. Antigen was retrieved using a pre-heated buffer of 10 mM citrate. To block endogenous peroxidase, slides were incubated in a 0.3% H_2O_2 solution. Subsequently, slides were incubated with 1 µg/mL anti-STING antibody (EPR13130, Abcam) or an isotype control (Rabbit IgG SP137, Abcam) in PBS solution with 1% BSA + 1% AB serum overnight at 4°C. Afterward, slides were incubated with a peroxidase-labeled polymer (Envision+ anti-rabbit, Dako), followed by a 3,3′-diaminobenzidine solution (DAB) to visualize specific signal. Slides were counterstained with hematoxylin and thereafter dehydrated and mounted. Images were obtained using a NanoZoomer 2.0-HT multi slide scanner (Hamamatsu Photonics). Hematoxylin and eosin (H&E) staining was performed using a standard technique.

Per patient, three cores were assessed. Patients that did not have at least two out of three cores with more than 20% tumor epithelium were excluded from the study (n = 173), resulting in a remaining total of 506 patients. Positivity of STING was analyzed by estimating the percentage of tumor epithelium at each staining intensity level (0 = negative, 1 = low, 2 = intermediate and 3 = high) and, subsequently, a Histo-score was assigned using the formula: [0× (% tumor epithelium 0+) + 1 × (% tumor epithelium 1+) + 2 × (% tumor epithelium 2+) + 3 × (% tumor epithelium 3+)].[28,29] This score, ranging from 0 to 300, was averaged over the 3 (or 2) cores of one patient. STING positive immune cells were only observed in a small subset of cores and, therefore, not included in the analysis. All TMA slides were scored by two individuals who were blinded for clinicopathological data. The interobserver agreement (intraclass correlation coefficient (ICC)) was 0.824. Scores were compared and if a difference of more than 50 was found, slides were reassessed.

Table 1. Relation of STING level to clinical characteristics.

	STING level – surgery cohort			STING level – RT/RCT cohort		
	<median (208.33)	**≥median (208.33)**	**P value**	**≤median (186.44)**	**>median (186.44)**	**P value**
Patients	123	128		128	127	
Age at diagnosis (y), continuous			0.524			0.471
Median	42.95	43.76		54.73	52.18	
Range	24.43–84.65	24.40–81.94		25.61–84.33	20.61–91.95	
HPV status (p16)			0.261			1,000
Positive	107	117		115	114	
Negative	11	5		9	9	
Unknown	5	6		4	4	
FIGO stage			0.077			0.747
IA2	-	-		-	-	
IB1	75	84		12	14	
IB2	21	29		16	10	
IIA	27	15		17	18	
IIB	-	-		59	65	
IIIA	-	-		3	2	
IIIB	-	-		19	14	
IVA	-	-		2	4	
Histology			**0.034**			**0.001**
Squamous carcinoma	74	94		95	116	
Adenocarcinoma	43	26		28	9	
Other	6	8		5	2	
Grade of differentiation			0.662			0.448
Good/moderate	70	80		67	74	
Poor/undifferentiated	51	46		53	43	
Other	2	2		8	10	
Lymphangioinvasion			0.467			0.990
No	54	50		72	76	
Yes	69	77		21	23	
Other	-	-		5	5	
Tumor diameter (cm)			0.937			0.901
0–4	88	91		31	33	
≥4	35	37		89	85	
Unknown	-	-		8	9	
Treatment			0.312			0.663
RH	77	71		-	-	
RH + post operative RT	38	51		-	-	
RH + post operative RCT	8	6		-	-	
Primary RT	-	-		58	61	
Primary RCT	-	-		70	66	
Follow-up (y), continuous			0.542			0.381
Median	5.35	5.93		3.68	3.80	
Range	0.31–19.35	0.53–21.31		0.14–16.28	0.17–18.36	
Result last follow-up			**0.014**			**0.041**
No evidence of disease	93	106		51	62	
Evidence of disease	2	0		2	0	
Death other cause	1	7		10	18	
Death of disease	27	15		65	47	

Abbreviations: FIGO: International Federation of Gynecologists and Obstetricians
Histology other: small cell carcinoma or unknown
RH: radical hysterectomy
RT: radiotherapy
RCT: radio-chemotherapy

TCGA

TCGA RSEM normalized mRNAseq and clinical data were downloaded from FireBrowse (http://firebrowse.org) on August 22, 2016. mRNA data were log2 transformed. After removal of normal tissue controls, 306 cervical cancer cases were informative for this study. Rows without gene names ("?") were removed. Histological subtypes were reclassified as follows: "Endocervical adenocarcinoma of the usual type", endocervical type of adenocarcinoma", endometrioid adenocarcinoma of endocervix" and "mucinous adenocarcinoma of endocervical type" were classified as adenocarcinoma (n = 47), squamous (n = 254) and adenosquamous (n = 6) were not reclassified. *STING1* (*TMEM173* in TCGA) levels were extracted per histological subtype. Stage of disease, i.e. early or late, was categorized according to FIGO stage of disease, with stage I – stage IB1 being early stage of disease and stage IB2- stage IVB as late stage. Patients with stage IB not further specified as A or B (n = 38) were removed for this part of the analysis, as were patients with no information on FIGO stage (n = 7). For survival analysis, patients were dichotomized based on median TMEM173 or ITGAE expression, respectively. Survival analyses were performed with the survminer package in R (version 0.4.6). Heatmaps of STING and immune-related genes were constructed with ComplexHeatmap package (version 2.2.0) and Circlize package (version 0.4.9) in R.

Statistics

Interobserver agreement of STING scoring was analyzed using the ICC based on a single measurement, absolute agreement, two-way mixed models. The differences between i) STING and CD103 levels for the surgery versus RT/RCT group, ii) CD103 based on below and above median STING per group, iii) *STING1* mRNA expression based on non-cancer and cervical cancer samples (TCGA data) and iv) *STING1* mRNA expression based on early and (locally) advanced stage cervical cancer samples (TCGA data) were assessed with Mann–Whitney U tests. The differences between i) STING based on histology per group and ii) *STING1* mRNA expression in cervical cancer samples based on histology (TCGA data) were assessed by Kruskal–Wallis tests with post hoc Dunn's multiple comparison test. Correlation plots were made and analyzed using GraphPad Prism version 8. Differences in clinicopathological characteristics regarding below versus above median STING per group were assessed by crosstab analyses with Chi-Square tests. DSS, disease-free survival (DFS) and survival probability (TCGA data) were plotted by Kaplan–Meier function and statistically assessed by Log Rank testing. Differences between DSS based on clinicopathological characteristics and STING were analyzed using Cox regression testing on DSS. Clinicopathological characteristics were included in multivariate analyses when p-value was <0.05 in univariate analysis. For the RT/RCT group, the number of patients with FIGO stage IIIA was too small for this analysis (non-determinable). All tests were performed two-sided and outcomes were considered significant when the p-value was <0.05. All statistical analyses were performed using IBM SPSS Statistics software version 23.0, GraphPad Prism version 8 or R version 3.6.2.

Results

Adenocarcinomas and advanced stage disease associate with lower levels of STING

STING expression has been reported to be lost or downregulated in multiple cancer types, and loss of STING was associated with poor prognosis.[14–18]To investigate the prognostic value of STING in cervical cancer patients, we assessed STING protein levels by immunohistochemistry (IHC) on tissue microarrays (TMA) of pretreatment cervical cancer biopsies. In line with previous work[30], STING expression was detected in the cytoplasm and perinuclear compartments, and present in both tumor cells and in isolated immune cells (Figure 1). Patients were included when at least two out of three cores contained more than 20% tumor epithelium. Based on this criterion, 506 patients were finally included. A high degree of uniformity in staining intensity was observed within and between the individual cores (Figure 1 and supplemental figure S3). This indicates that the staining pattern in most tumors appears to be homogeneous and that our TMA sufficiently captures STING protein levels in cervical cancer patients. Comparison of STING values of early stage disease (FIGO 1B1) versus (locally) advanced disease stages (FIGO IB24A) revealed that STING is significantly lower in the latter group (Supplemental figure S4). As choice of treatment depends on FIGO stage, patients were divided into two groups based on their primary treatment being surgery (n = 251) or radio(chemo)therapy (RT/RCT) (n = 255). In line, we observed that STING levels were significantly lower in the RT/RCT group (median 186.44) compared to the surgery group (median 208.33) (Figure 2a, $p < .0001$). In addition to treatment, each group was dichotomized based on low or high STING, defined as below or equal to/above median STING level per group to avoid bias evoked by the relation of STING with FIGO, and analyses of clinical characteristics were performed to assess whether STING was associated with disease progression (Table 1). Most clinical characteristics such as age at diagnosis, p16 status as surrogate marker for HPV status, grade of differentiation, lymphangioinvasion, tumor diameter and years of follow-up were found to be consistent between low and high STING. Interestingly, the histological subtype distributions were significantly different between patients with low and high STING ($p = .033$ surgery and $p < .001$ RT/RCT group). Further assessment revealed that STING levels were significantly lower in adenocarcinomas as compared to squamous cell carcinomas in both groups (Figure 2b, $p = .0003$ surgery and $p < .0001$ RT/RCT group). We did not observe significant differences in STING levels between cervical adenocarcinoma histological subtypes (Supplemental table S1). Patients with adenocarcinomas tended to have worse outcome than patients with squamous or other tumor types, but these differences did not reach statistical significance (Supplemental figure S5A, $p = .332$ surgery and 5B, $p = .060$ RT/RCT). Previously, it was shown that HPV- cervical carcinomas are more frequently adenocarcinomas and seem to be associated with worse survival.[31,32] In the present study, DSS for patients with p16- adenocarcinomas was indeed worse than for patients with p16+ adenocarcinomas in the RT/RCT cohort ($p = .012$, Log Rank test), but only regarded two p16- patients. p16 status did not affect STING level in adenocarcinomas ($p = .269$ surgery and $p = .676$ RT/RCT, Chi-Square test).

STING is prognostic for survival of cervical cancer patients

The status at last follow-up significantly differed between patients with low and high STING in both groups (Table 1, $p = .014$ surgery and $p = .041$ RT/RCT group). Here, we observed that patients with low STING showed higher recurrence rates, more residual disease and increased disease-specific mortality compared with that of patients with high STING expression. As expected, having more advanced stage disease, patients included in the RT/RCT group had worse outcomes. Specifically, 112/255 RT/RCT group patients (43.92%) died of disease versus 42/251 (16.73%) in the surgery group. To follow up on these findings, we further explored the relation between STING and survival. As we determined the levels of STING in pretreatment material, we first assessed disease-specific survival (DSS) based on below and above median STING for all 506 patients (adjusted median of 200). High STING strongly associated with improved outcome over patients with low STING levels, irrespective of subsequent therapy (Supplemental figure S6, $p = .000$). In line, for both the surgery and the RT/RCT groups, we observed that DSS was significantly worse for patients with low STING (Figure 2c, $p = .029$ surgery, and

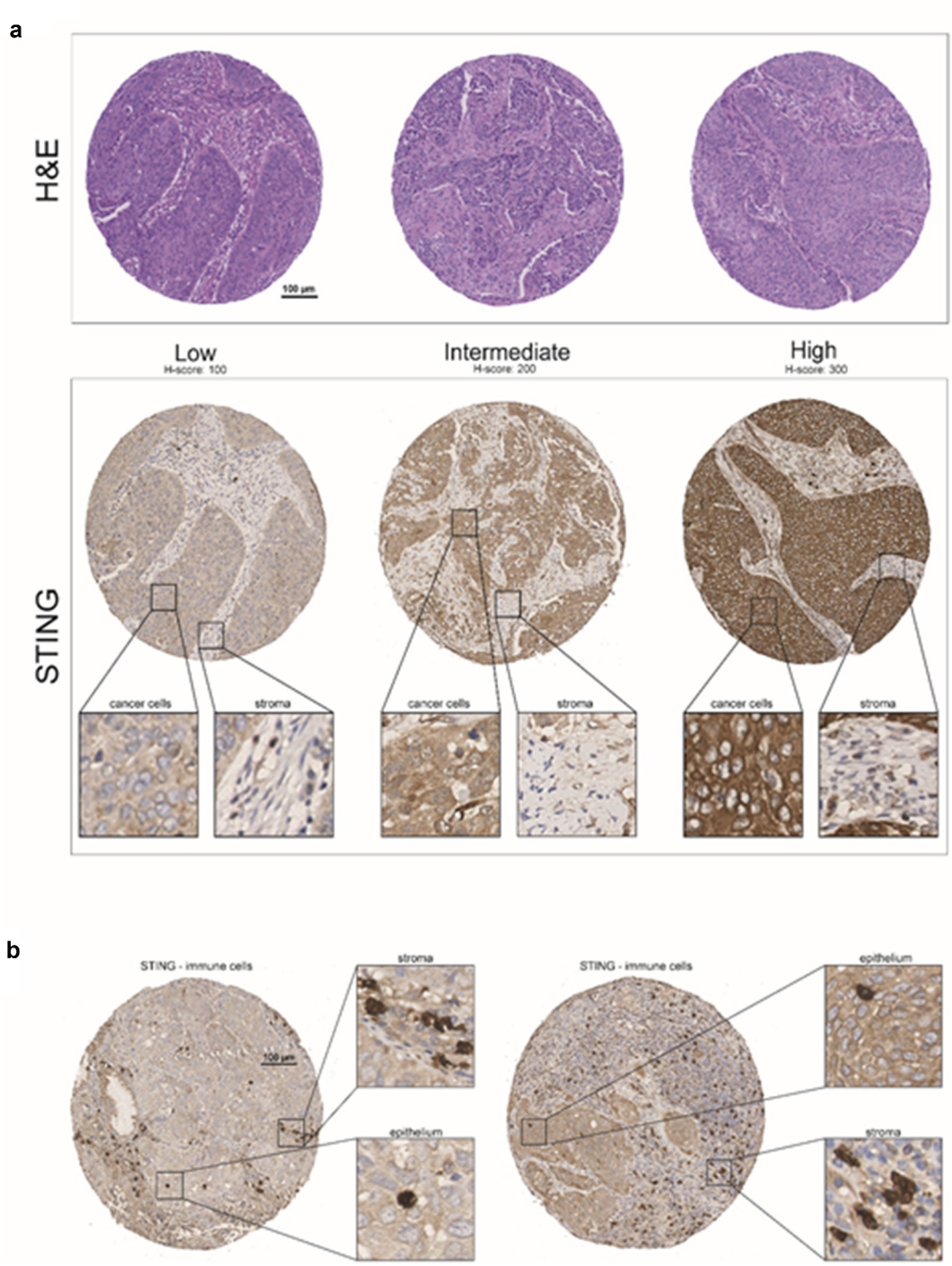

Figure 1. Representative images of H&E stainings and immunohistochemical STING stainings in cervical carcinomas. A) tissue microarray (TMA) cores representing tissue with low (H-score: 100), intermediate (H-score: 200) and high (H-score: 300) STING levels, based on intensity of DAB (brown). For each core magnifications of areas with cancer cells and stroma are depicted. B) TMA cores representing STING protein positivity in tumor infiltrating immune cells. For each core magnifications of areas with epithelium and stroma are depicted. Images were obtained using a NanoZoomer 2.0-HT multi slide scanner (Hamamatsu Photonics).

2D p = .046 RT/RCT group). Low STING was also significantly prognostic for worse disease-free survival (DFS) in both groups (Figure 2e p = .039 surgery, and 2 F, p = .026 RT/RCT group). We conclude that STING levels associated with prognosis of cervical cancer patients, in both early stage and locally advanced stage disease. Specifically, low levels of STING predicted worse survival outcome.

Multivariate analyses confirms the independent prognostic value of STING in cervical cancer

To examine if the high prognostic value of STING in cervical cancer is independent of other factors, we performed univariate and multivariate Cox regression analyses on both groups (Tables 2 surgery and Tables 3 RT/RCT group). For the surgery

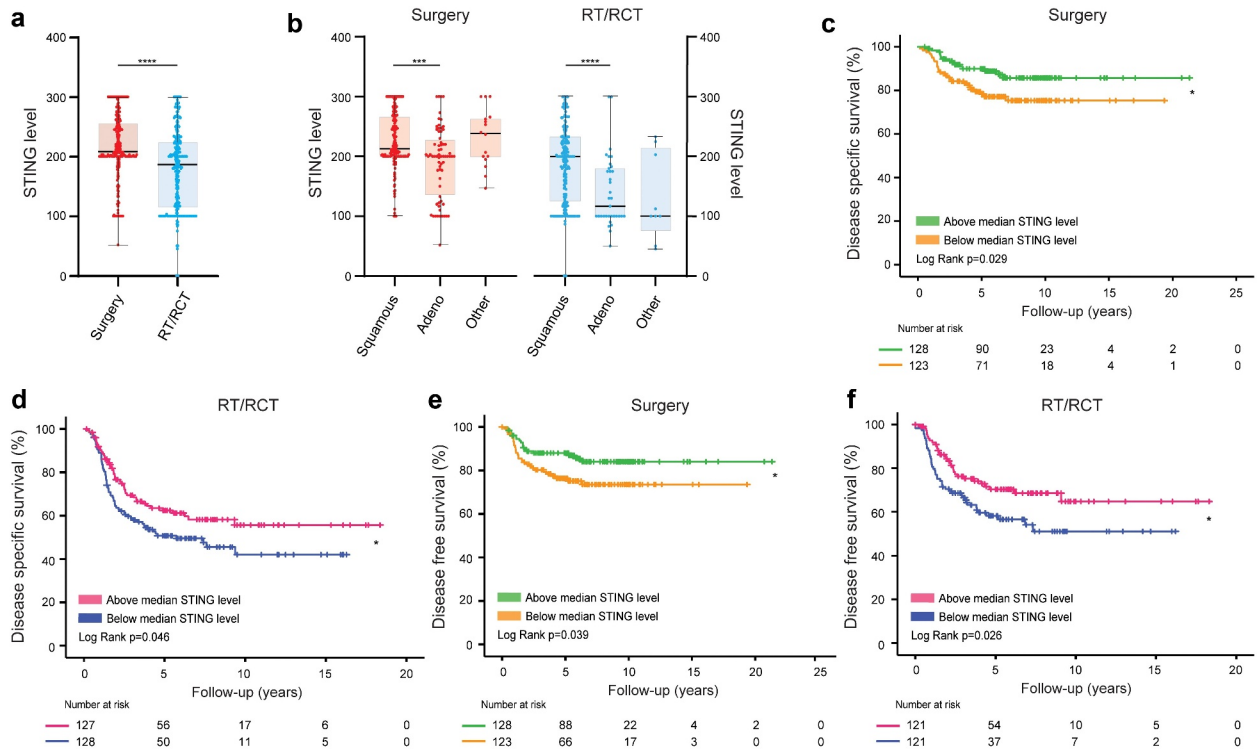

Figure 2. STING is prognostic for survival of cervical cancer patients. A) STING scores per group, surgery (red) or RT (blue). B) STING scores group, surgery (red) or RT (blue), based on histological subtype. A, B) each dot represents one patient. Median, quartiles and maximal values are depicted by boxplots. Statistical analyses, either by Mann–Whitney U or Kruskal–Wallis with post hoc Dunn testing, were performed using GraphPad Prism version 8. A *p* value of <.05 was considered statistically significant. C, D) Disease specific survival in years for the C) surgery and D) RT/RCT group. E, F) Disease free survival in years for the E) surgery and F) RT/RCT group. Orange and blue curves represent patients with STING levels below the median level; green and pink curves patients with STING levels above the median. All STING levels were obtained from immunohistochemical STING stainings on TMA. All four statistical analyses were performed by Log Rank testing in SPSS. A *p* value of <.05 was considered statistically significant. Numbers at risk are depicted below each Kaplan Meier plot and correspond to the years of follow-up at the y-axis.

Table 2. Surgery cohort. Cox regression survival analyses.

Disease-specific survival, Enter for univariate and Forward Stepwise (LR) for multivariate, n = 251								
	Univariate analyses				Multivariate analysis			
	HR	*95% CI*		*p-value*	*HR*	*95% CI*		*p-value*
Age at diagnosis (years)								
<54 y	ref	ref	ref	ref				
>54 y	2,098	1,123	3,919	**0.020**				
FIGO stage								
IB1	ref	ref	ref	ref	ref	ref	ref	ref
IB2	2,139	1,010	4,531	**0.047**	1,878	0.864	4,084	0.112
IIA	3,149	1,542	6,430	**0.002**	2,744	1,337	5,632	**0.006**
Histology								
Squamous carcinoma	ref	ref	ref	ref				
Adenocarcinoma	1,694	0.900	3,190	0.103				
Other	0.942	0.223	3,988	0.936				
Differentiation grade								
Good/moderate	ref	ref	ref	ref				
Poor/undifferentiated	1,657	0.898	3,058	0.106				
Other	1,626	0.218	12,122	0.635				
Lymphangioinvasion								
No	ref	ref	ref	ref	ref	ref	ref	ref
Yes	3,830	1,697	8,641	**0.001**	3,831	1,694	8,662	**0.001**
Tumor diameter (cm)								
0–4	ref	ref	ref	ref				
≥ 4	2,303	1,254	4,229	**0.007**				
STING_median								
Low	ref	ref	ref	ref	ref	ref	ref	ref
High	0.501	0.267	0.942	**0.032**	0.456	0.237	0.879	**0.019**

FIGO: International Federation of Gynecology and Obstetrics
Histology other: small cell carcinoma or unknown

group, these analyses revealed that univariate prognostic factors for DSS were age at diagnosis, FIGO stage, lymphangioinvasion, tumor diameter and STING level (based on above and below median level). After multivariate analysis including these factors, only FIGO stage, lymphangioinvasion and STING level were found to be independently prognostic. For the RT/RCT

Table 3. RT/RCT cohort. Cox regression survival analyses.

Disease-specific survival, Enter for univariate and Forward Stepwise (LR) for multivariate, n = 255								
	Univariate analyses				Multivariate analysis			
	HR	*95% CI*		*p-value*	*HR*	*95% CI*		*p-value*
Age at diagnosis (years)								
<54 y	ref	ref	ref	ref				
>54 y	0.875	0.603	1270	0.483				
FIGO stage								
IB1	ref	ref	ref	ref	ref	ref	ref	ref
IB2	2,550	0.659	9,864	0.175	1,843	0.439	7,730	0.403
IIA	4,989	1,462	17,025	**0.010**	3,751	1,059	13,295	**0.041**
IIB	4,831	1,515	15,404	**0.008**	4,363	1,357	14,022	**0.013**
IIIA	ND	ND	ND	ND	ND	ND	ND	ND
IIIB	7,590	2,251	25,527	**0.001**	7,952	2,315	27,319	**0.001**
IVA	15,483	3,696	64,849	**<0.0001**	13,227	2,618	66,882	**0.022**
Histology								
Squamous carcinoma	ref	ref	ref	ref				
Adenocarcinoma	1,673	1,025	2,616	**0.039**				
Other	1,701	0.624	4,637	0.300				
Differentiation grade								
Good/moderate	ref	ref	ref	ref				
Poor/undifferentiated	1,353	0.927	1,975	0.117				
Other	0.481	0.175	1,327	0.158				
Lymphangioinvasion								
No	ref	ref	ref	ref				
Yes	1,767	1,115	2,801	**0.015**				
Other	1,108	0.401	3,058	0.844				
Tumor diameter (cm)								
0–4	ref	ref	ref	ref				
≥ 4	1,656	1,035	2,649	**0.035**				
Unknown	0.921	0.373	2,273	0.859				
STING_median								
Low	ref	ref	ref	ref	ref	ref	ref	ref
High	0.684	0.470	0.996	**0.048**	0.597	0.384	0.928	**0.022**

FIGO: International Federation of Gynecology and Obstetrics
ND: not determinable
Histology other: small cell carcinoma or uknown

group, FIGO stage, histology, lymphangioinvasion, tumor diameter and STING level were univariate prognostic factors for DSS. Multivariate analysis revealed that FIGO stage and STING level were independently prognostic for survival. Together, the analyses show that STING is an independent prognostic factor for survival in cervical cancer.

High STING levels combined with CD103-positive tumor infiltrating lymphocytes strongly associates with improved prognosis

We have previously reported that CD103-positive tumor-infiltrating lymphocytes are tumor-reactive, intraepithelial CD8-positive T cells that are associated with prognostic benefit and therapy response in cervical cancer.[23] Since STING pathway activation can play a role in the induction of CD8+ T cell responses against tumor-derived antigens *in-vivo*[8], we wondered whether CD103+ TIL infiltration associates with STING levels. For this reason, we assessed CD103+ TIL infiltration in cervical cancer patients[23] in combination with STING scores. CD103+ TIL infiltration scores were available for 216 patients in the surgery group and 232 patients in the RT/RCT group. Levels of STING correlated with levels of CD103 (r = 0.2323, $p < .0001$), largely due to patients of the RT/RCT cohort (Supplemental figure S7A-C). Off note, no correlation between CD8 and STING levels was found (Supplemental figure S7D-F). Kaplan–Meier plots of DSS and DFS were generated to assess the correlation of STING and CD103+ TIL infiltration with survival. The cutoff was determined based on median STING and median CD103 + TIL infiltration for each group, resulting in four subgroups: $STING^{high}/CD103^{high}$, $STING^{high}/CD103^{low}$, $STING^{low}/CD103^{high}$ and $STING^{low}/CD103^{low}$. Patients in the surgery group appeared to have significantly more CD103 infiltration than patients from the RT/RCT group (Figure 3a, <.0001). When further exploring the difference per group with regard to STING, we observed significantly higher CD103 infiltration in patients with high STING than in patients with low STING in both groups (Figure 3b, $p = .0242$ surgery and $p < .0001$ RT/RCT group). In contrast to STING, CD103 was only prognostic for DSS and DFS of patients in the RT/RCT group, thus with locally advanced disease (Supplemental Figure S8B $p = .014$ and Figure 8D $p = .021$, respectively). Combining the factors STING and CD103 revealed significant prognostic value for survival of patients from the RT/RCT group (Figures 3d and 3f, $p = .014$ and $p = .004$, respectively). Specifically, patients characterized by a $STING^{high}/CD103^{high}$ pattern had a longer DSS and DFS than patients from the $STING^{low}/CD103^{low}$ group. Similar to the observation that CD103 did not have prognostic value (DSS and DFS) in the surgery group (Supplemental Figure S8A and 8C, $p = .280$ and $p = .690$ resp.), the combination of CD103 and STING was also not prognostic in this group (Figures 3c and 3e, $p = .154$ and $p = .148$ resp.). The observed effects were independent of

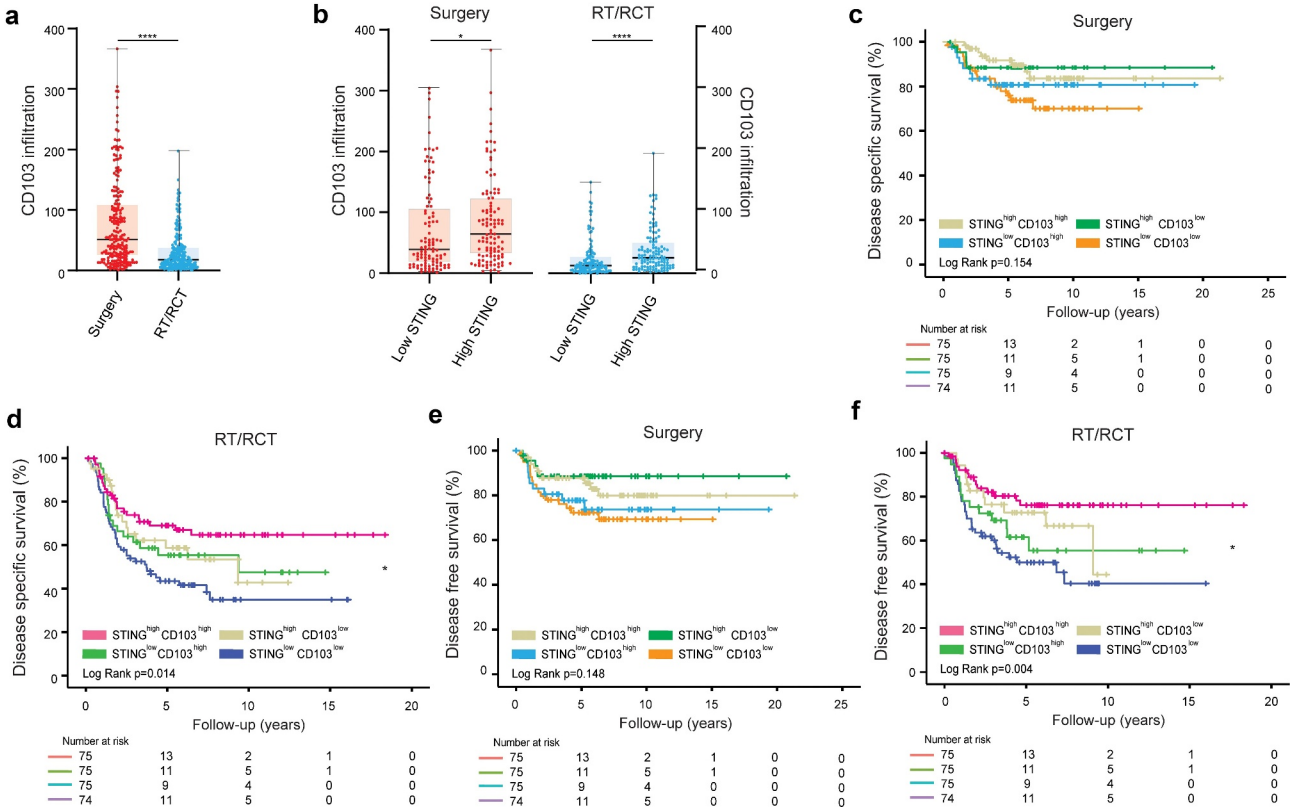

Figure 3. High STING and CD103 is strongly associated with improved prognosis. A) CD103 scores per group, surgery (red) or RT (blue). B) CD103 scores per group, surgery (red) or RT (blue), stratified for low or high STING levels. A, B) each dot represents one patient. Median, quartiles and maximal values are depicted by boxplots. Mann–Whitney U statistical analyses were performed using GraphPad Prism version 8. A p value of <.05 was considered statistically significant. C, D) disease specific survival in years for the C) surgery and D) RT/RCT group. E, F) disease free survival in years for the E) surgery and F) RT/RCT group. Colors represent specific combinations of STING and CD103 (above and/or below median levels) and are clarified in the legend in each plot. All STING levels were obtained from immunohistochemical STING stainings on TMA, CD103 levels were obtained previously by immunohistochemical TMA staining[23]. All four statistical analyses were performed by Log Rank testing in SPSS. A p value of <.05 was considered statistically significant. Numbers at risk are depicted below each Kaplan Meier plot and correspond to the years of follow-up at the y-axis.

HPV infection, as CD103 levels were comparable for p16 + and p16- patients (p = .621 surgery and p = .359 RT/RCT, Chi-Square test).

STING1 expression does not correlate to survival in TCGA samples from patients with cervical cancer

Lastly, we wanted to further explore our observations by investigating mRNA data of cervical cancer from the TCGA database. *STING1* mRNA expression levels did not differ between non-cancerous tissue (n = 3) and cervical cancer tissue (n = 306) of patient samples in the TCGA database (Figure 4a, p = .1245). Also, *STING1* expression levels did not differ between non-cancerous tissue (n = 3), adenocarcinoma (n = 47) and squamous cell carcinoma (n = 253) in cervical cancer patients (Figure 4b, p = .2946). Since treatment data was often missing, we separated patient samples from the TCGA database into early (FIGO I–IB1) and (locally) advanced stage disease (FIGO IB2 to IVB). Based on standard-of-care treatment regimens, the early and late stage disease represent our surgery and RT/RCT groups, respectively. In contrast to the observations in our IHC data in these groups, TCGA *STING1* mRNA levels were comparable for cervical cancer patients based on early (n = 86) and (locally) advanced stage disease (n = 176) (Figure 4c, p = .5609). *STING1* mRNA expression had no prognostic value when looking at overall survival of all cervical cancer patients (Figure 4d, p = .105) and of patients with early stage disease (Figure 4e, p = .4) or (locally) advanced stage disease (figure 4f, p = .6). In contrast, the combination of *STING1* and *ITGAE* (encoding CD103) expression was prognostic for survival (Figure 4g, p = .048). However, this was likely mainly due to the strong, positive correlation between *ITGAE* expression and survival, as our group previously published.[23] Lastly, we assessed *STING1* expression in cervical cancer patients from the TCGA database in relation to mRNA levels of important immunological markers such as *ITGAE, CD8A, GZMB*, cytokines and well-known immune checkpoints such as *PDCD1, LAG-3* and *TIGIT* (Figure 4h). Notably, *ITGAE* expression did not correlate with *STING1* expression (R = 0.0335, p = .1062). On the other hand, expression of *STING1* correlated significantly, although fairly weakly, to expression of many of the other immunological markers, including *CD8A*, effector genes *PRF1* (R = 0.1902, p < .0001) and *GMZB* (R = 0.1049, p = .0005), immune checkpoints *PDCD1* (R = 0.1792, p = .0003) and *TIGIT*

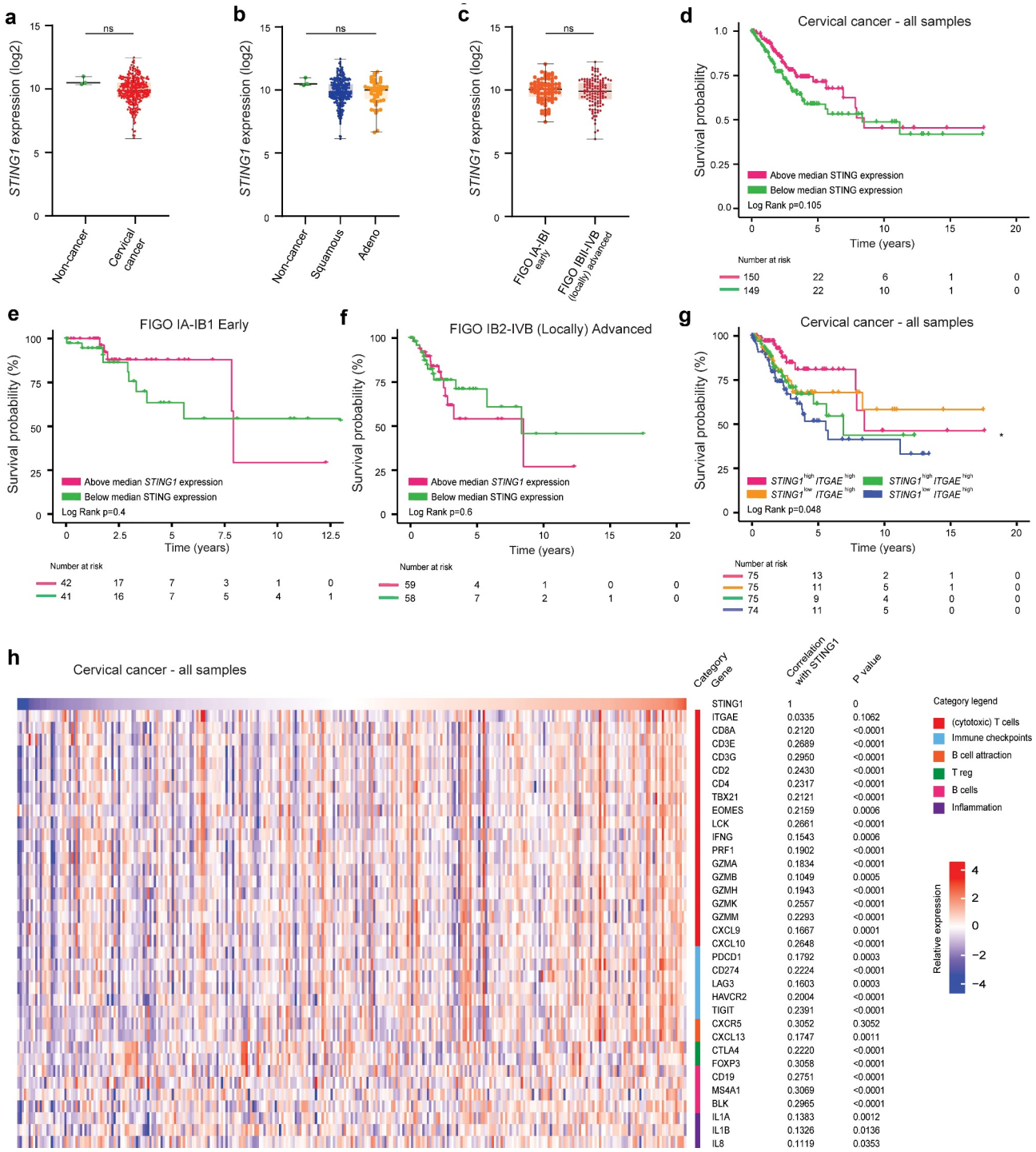

Figure 4. Correlation between STING1 expression and survival in TCGA samples from patients with cervical cancer. All expression and survival data were derived from cervical cancer samples (n = 306) in the TCGA database. The mRNA expression data were log2 transformed before analysis. A) *STING1* mRNA expression scores in non-cancer samples (green, n = 3) and cervical cancer samples (red). B) *STING1* mRNA expression scores in non-cancer samples (green) and cervical cancer samples based on histological subtype (squamous cell carcinoma in blue, n = 253 and adenocarcinoma in orange, n = 47). C) *STING1* mRNA expression scores in cervical cancer samples, stratified for early FIGO stage (orange, IA to IB1, n = 86) and (locally) advanced FIGO stage (red, IB2-IVB, n = 176). A, B, C) each dot represents one patient. median, quartiles and maximal values are depicted by boxplots. Statistical analyses, either by Mann–Whitney U or Kruskal–Wallis with post hoc Dunn testing, was performed using GraphPad Prism version 8. A *p* value of <0.05 was considered statistically significant. D, E, F) Survival probability plots in years based on above (red) and below (blue) median *STING1* expression in D) all cervical cancer samples, E) cervical cancer samples annotated as early FIGO stage and F) cervical cancer samples annotated as (locally) advanced FIGO stage (red, IB2-IVB). G) survival probability based on four above and below median expression combinations of *STING1* and *ITGAE* (encoding CD103), for which color annotations are clarified in the legend. All four statistical survival analyses were performed by Log Rank testing in SPSS. A *p* value of <0.05 was considered statistically significant. Numbers at risk are depicted below each Kaplan Meier plot and correspond to the years of follow-up at the y-axis. H) Relative gene expression of (cytotoxic) T cells (red), immune checkpoints (blue), B cell attraction (orange), T regulatory T cells (T reg)(green), B cells (pink) and inflammation (purple) associated genes in TCGA data of cervical cancer. Samples were ranked by *STING1* expression. Red indicates high relative expression, and blue indicates low relative expression of the indicated gene. Correlations of gene expression with *STING1* expression were determined by Spearman correlation testing using R software.

(R = 0.2391, $p < .0001$), and B-cell attracting chemokine *CXCL13* (R = 0.1747, $p = .0011$). When further exploring this relation by separating the patients based on histology, a similar pattern was only observed for squamous cell carcinomas but not adenocarcinomas (Supplemental Figure S9).

Discussion

In this study we show that high STING protein level is associated with improved survival in cervical cancer patients primarily treated with surgery or with (chemo)radiation therapy (RT/RCT group). Importantly, combining STING levels together with the prognostic factor CD103+ TIL infiltration strongly associated with improved survival in the RT/RCT group. The prognostic value of pretreatment STING was independent of subsequent therapeutic modality. Nevertheless, patients in the RT/RCT group had significantly lower levels of STING pretreatment than patients in surgery group, the latter having a better prognosis due to more early stage of disease. It was previously reported for gastric cancer and hepatocellular carcinoma patients that STING protein levels were decreased in tumor tissues compared to non-tumor tissues and inversely correlated with tumor stage.[15,33] In line, low STING associated with poor prognosis in multiple cancer types.[14–19] We suggest that STING signaling may be highly deficient in patients with (locally) advanced stage disease, which could lead to poor anti-tumor immunity, immune evasion by the tumor and more progressive disease. In accordance, patients in the RT/RCT group with low STING showed significantly worse outcomes than patients with high STING levels. These findings may also support the hypothesis that STING signaling is important for radiation-mediated anti-tumor immunity in immunogenic tumors[34], in which DNA exonuclease Trex1 may be an important regulator.[35] The importance of STING is supported by the finding that patients in the surgery group with low STING had significantly worse outcomes than patients with high STING levels. Of note, it would be interesting to investigate the potential role of HPV oncogene E7 protein expression in STING expression in these patients, as E7 is described to act antagonistically in the cGAS-STING pathway and it can promote autophagy-dependent degradation of STING.[19] In both groups, patients with adenocarcinomas appeared to have lower STING levels than patients with squamous cell carcinomas. Moreover, the patients with low STING levels in both groups had significantly worse outcomes than patients with high STING levels. It is reported that cervical cancer patients with adenocarcinomas have worse survival than patients with squamous cell carcinomas.[36,37] We speculated that this may be partially explained by having lower STING levels. In our groups, histology did not significantly associate with survival, although there was a trend toward adenocarcinomas having worse survival compared to other tumor types. In gastric cancer patients, STING expression was decreased in both low and high stage tumors, indicating that reduced STING expression may already develop in early stages of gastric cancer.[14] STING protein levels have been investigated previously in high grade cervical intraepithelial neoplasia (CIN3).[20] This study showed that STING expression is induced in cervical dysplasia. This finding is in line with our observation that STING is expressed in cervical malignancies. Our data on STING levels is limited to cancerous tissue. Hence, based on our IHC data, no firm conclusions can be drawn regarding down-regulation of STING in cervical cancer. Analysis using mRNA data from the TCGA database indicated that *STING1* expression is similar for non-cancerous tissue and cervical cancer tissue, although the number of non-cancerous samples was limited and mRNA expression may not reflect protein levels as a result of differences in synthesis and turnover.[38] Therefore, it remains to be elucidated whether STING protein is truly down-regulated in cervical cancer as compared to healthy cervix tissue. Based on the function of STING, we speculate that STING expression is not a driver for malignant transformation, but a response to malignant transformation. As activator of innate immune recruitment via interferon induction after sensing of cytoplasmic DNA by cGAS, STING may suppress malignant transformation and low STING1 protein levels might therefore mark an increased risk for development of premalignant lesions to malignant lesions. Furthermore, TCGA database analysis showed a correlation between CD103 mRNA, but not STING mRNA, and outcome in cervical cancer. This finding was not reflected on protein level since STING associated with outcome in both our patient groups. The contradiction may indicate a discordance between mRNA levels and protein levels. Of note, we only included assessment of STING protein and mRNA levels, which might not necessarily reflect STING signaling activity. STING is part of the cGAS-STING pathway, which includes multiple components such as cGAS, IRF3 and TANK-binding kinase 1. In colorectal cancer cell lines for example, it was found that STING-dependent signaling is frequently suppressed through silencing of cGAS.[18] This lead to a reduction in type I IFN production upon DNA-damage, helping tumor cells to escape the immune-surveillance system. Therefore, it may be of interest to study other STING signaling pathway molecules in cervical cancer patients. STING is reported to be associated with infiltration of CD8+ T cells.[16,39] Previously, we and others have shown that integrin CD103 marks tumor-reactive CD8+ T cells in the tumor epithelium.[22–24,40,41] Interestingly, like STING, CD103 infiltration was significantly higher in patients with early stage disease than patients with more advanced stage disease. High CD103 infiltration, but not CD8, was associated with high levels of STING in both groups. Moreover, analysis of TCGA mRNA data indicated a significant, positive correlation between expression of *STING1* and expression of various T cell related genes, although not for *ITGAE* (encoding CD103). Based on these findings, we speculate that STING signaling may be important for infiltration of tumor-reactive T cells in cervical cancer, without affecting the total CD8 pool. Our findings are in line with previous work that linked STING with immune response.[42] Although high infiltration of CD103+ cytotoxic T cells is reported to be prognostic for improved outcome of patients with cervical cancer[23] and other cancer types,[40,43] in the current study we did not observe a significant association between CD103 protein and outcome for cervical cancer patients with surgery as first modality of treatment, nor for

CD103 individually nor for the combination of CD103 and STING. However, despite statistical insignificance, it appeared that in the surgery group, patients with STING^{high}/CD103^{high} pattern had better outcomes than patients with STING^{low}/CD103^{low} pattern. In addition, outcome for patients in the radiotherapy group was improved when STING expression was combined with CD103 infiltration. Since cervical cancer patients with low STING have inferior survival and protein STING levels associated with CD103 levels, we speculate that STING agonistic therapy may be beneficial for treatment of cervical cancer patients with defective or reduced STING signaling. Research is being conducted on finding an effective agonist for human STING as a potential new approach in cancer immunotherapy. These agonists include STING agonist formulated cancer vaccines[44], modified cyclic nucleotides for intra-tumoral administration[11], but also a nano-particle incorporated STING agonist for systemic delivery[45]. Thus far, STING agonists alone or in combination with other therapies such as irradiation, chemotherapy and blockade of immune-system checkpoints, including PD-1,[44–49] show promising preclinical results. In conclusion, our study demonstrated that STING is an independent prognostic factor for favorable survival in cervical cancer. Furthermore, a high STING level combined with high frequencies of CD103+ TIL infiltration strongly associated with improved prognosis in patients with late stage disease. Combining these prognostic factors may improve risk stratification of cervical cancer patients, independent from established clinical prognostic parameters, and aid in identifying patients with defective or reduced STING signaling. Activating the STING pathway in these patients may be therapeutically beneficial and should be considered in the treatment of cervical cancer.

Conflicts of interest:

The authors declare no potential conflicts of interest.

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