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ORIGINAL ARTICLE

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Association of homozygous variants of *STING1* with outcome in human cervical cancer

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Abstract

DNA-sensing receptor Cyclic GMP–AMP Synthase (cGAS) and its downstream signaling effector STimulator of INterferon Genes (STING) have gained significant interest in the field of tumor immunology, as a dysfunctional cGAS-STING pathway is associated with poor prognosis and worse response to immunotherapy. However, studies so far have not taken into account the polymorphic nature of the STING-encoding *STING1* gene. We hypothesized that the presence of allelic variance in *STING1* would cause variation between individuals as to their susceptibility to cancer development, cancer progression, and potential response to (immuno)therapy. To start to address this, we defined the genetic landscapes of *STING1* in cervical scrapings and investigated their corresponding clinical characteristics across a unique cohort of cervical cancer patients and compared them with independent control cohorts. Although we did not observe an enrichment of particular *STING1* allelic variants in cervical cancer patients, we did find that the occurrence of homozygous variants HAQ/HAQ and R232H/R232H of STING1 were associated with both younger age of diagnosis and higher recurrence rate. These findings were accompanied by worse survival, despite comparable mRNA and protein levels of STING and numbers of infiltrated $CD8^+$ T cells. Our findings suggest that patients with HAQ/HAQ and R232H/R232H genotypes may have a dysfunctional cGAS-STING pathway that fails to promote efficient anticancer immunity. Interestingly, the occurrence of these genotypes coincided with homozygous presence of the V48V variant, which was found to be individually associated with worse outcome. Therefore, we propose V48V to be further evaluated as a novel prognostic marker for cervical cancer.

KEYWORDS

allelic variants, cervical cancer, human papillomavirus, interferon signaling, STING/TMEM173

Nijman and de Bruyn authors share senior authorship.

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1 | INTRODUCTION

Cervical cancer is the fourth most common type of cancer in women worldwide and the leading cause of cancer deaths in over 50 countries.[1] A persistent human papillomavirus (HPV) infection underlies the development of cervical cancer.[2] In recent years, several genomewide association studies have led to the discovery of multiple genes influencing the susceptibility to cervical cancer.[3] Allelic variants of these genes together may explain up to 24% of the variance in the risk for developing cervical cancer.[4] One of the genes is *STING1* (also known as *TMEM173* and *MITA*), encoding STimulator of INterferon Genes (STING), which is a downstream signaling effector of the DNA-sensing receptor Cyclic GMP–AMP Synthase (cGAS). During infections, cGAS detects DNA from pathogens in the cytosol of the cell and subsequently elicits STING activation and ultimately induction of interferon type I (IFN-I) signaling, thereby provoking innate and, subsequently, adaptive immunity.[5]

In the context of cancer, the cGAS-STING pathway can identify chromosomal instability in cancerous cells by detecting cytoplasmic DNA[6-8] or identify infection with potentially carcinogenic viruses by detecting viral DNA, such as DNA from HPV.[9] In addition, it is reported to improve the efficacy of (DNA damage–inducing) radiotherapy.[10,11] Several studies have shown that dysfunction of the cGAS-STING pathway, caused for instance by deficient STING translocation to the Golgi and decreased expression levels of cGAS and STING, leads to poor IFN-I production.[10,12-16] In multiple cancer types such as breast, gastric, and hepatocellular carcinoma, levels of STING were found to be decreased compared with healthy tissue.[14-16] In another study regarding DNA damage, loss of STING hampered tumor regression upon application of multiple therapeutic strategies such as immune checkpoint blockade.[8] As loss and decreased levels of STING are associated with poor prognosis,[15-17] the potential therapeutic benefit of inducing STING is currently under investigation.[18] However, the existence of allelic variants of the STING gene may complicate the efficacy of this treatment modality because some variants cause an inherent defect in STING functionality.[19,20] A total of 76 biallelic variants are observed in the STING-encoding *STING1* gene. Of these, 26 are located in the coding sequence. Moreover, six represent synonymous substitutions, among which is rs7447927 (V48V, c.144C > G).[21] The most common variants in *STING1* are the nonsynonymous rs1131769 (R232H, c.695G > A), rs11554776 (R71H, c.212G > A), rs78233829 (G230A, c.689G > C), and rs7380824 (R293Q, c.878G > A).[21,22] In combination, the latter three are termed the HAQ genotype, which occurs in 20.4% of the human population.[21] The R232H, G230A, and R293Q substitutions are located in the CDN-binding region of the gene and cause defective response of STING.[23] For instance, a recent study reported on the effect of the homozygous HAQ variant of STING in individuals infected with human immunodeficiency virus (HIV), stating that it contributes to reduced levels of IFN production and a reduced immune response.[24]

In the context of HPV infection, we hypothesized that allelic variants of *STING1* may increase the susceptibility of patients to persistent infection and thereby risk of cervical cancer development. Furthermore, we hypothesized that the occurrence of particular variants may affect immunity against established cervical cancer. To investigate this, we comprehensively defined the variance in STING by genotyping *STING1* and assessing CD8$^+$T cell infiltration across a large cohort of cervical cancer patients.

2 | MATERIALS AND METHODS

2.1 | Patient cohort

All patients visiting the outpatient clinic of the University Medical Center Groningen (UMCG, the Netherlands) for diagnostics or treatment of cervical neoplasia or nonmalignant reasons (such as uterovaginal prolapse or uterine myomas) were asked to participate. After informed consent, frozen tissue samples and cervical epithelial scrapings were collected. The cervical scrapings cells were suspended in 250 μL of 4M guanidium isothiocyanate (GT) and frozen at −80°C. Frozen tissue was embedded with Tissue-Tek® OCT™ Compound (Sakura Finetek Europe BV) and stored at −80°C. RNA was isolated using Ambion TRIzol Reagent (Invitrogen) or by chloroform/isopropanol precipitation. cDNA was synthesized using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT). Data of healthy controls, patient and tumor characteristics, and clinical follow-up data were collected retrospectively in an anonymized database (see also document S1 for detailed experimental procedures). Ethical approval for the study was provided by the medical ethics review committee (METc) of the UMCG (study number 201800288).

2.2 | Analysis of STING1 expression and allelic variance

STING1 cDNA was isolated through PCR amplification, followed by gel extraction and Sanger sequencing. The reference sequences of the *STING1* mRNA transcript were obtained online from National Center for Biotechnology Information Gene.[25] To determine prevalence of allelic variants, data from the NCBI 1000 Genomes phase 3 browser[26] were analyzed, containing data from 2504 whole genomes (5008 genotypes), of which 503 (1006 genotypes) were from European donors (107 Spanish, 107 Italian, 99 Finnish, 91 British, and 99 Utah residents with a European background). This population is further referred to as European reference cohort and used as additional reference for the allele and genotype frequencies in the current study population. The following six *STING1* single-nucleotide substitutions were included for analysis: V48V, R71H, G230A, R232H, R293Q, and A313T. Somatic substitutions such as R284M and R284G were not taken into account, as it was formerly reported that the mutation rate in *STING1* is only 0.11%.[23] Combinations of single-nucleotide substitutions were annotated either as HAQ genotype (V48V, R71H, G230A, and R293Q), R232H genotype (V48V and R232H), or AQ genotype (V48V, G230A,

TABLE 1 Distribution of STING1 genotypes across patient and control cohorts

	Patient cohort n = 150	Healthy controls n = 20	European reference n = 503
WT/WT	87 (58.0)	11 (55.0)	258 (51.3)
WT/HAQ	27 (18.0)	3 (15.0)	97 (19.3)
WT/R232H	22 (14.7)	4 (20.0)	88 (17.5)
WT/AQ	1 (0.7)	-	3 (0.6)
WT/A313T	-	1 (5.0)	4 (0.8)
WT/G230A	-	-	1 (0.2)
HAQ/HAQ	5 (3.3)	-	14 (2.8)
HAQ/R232H	3 (2.0)	1 (5.0)	22 (4.4)
R232H/R232H	4 (2.7)	-	14 (2.8)
R232H/AQ	1 (0.7)	-	-
HAQ/AQ	-	-	1 (0.2)
AQ/AQ	-	-	1 (0.2)

and R293Q); A313T was not observed in the patient cohort. Expression analysis of *STING1* was performed by qRT-PCR (see also document S1 for detailed experimental procedures).

2.3 | Immunohistochemistry

Whole tumor tissue sections of 89 and 99 included cervical cancer patients were stained for STING and CD8, respectively. Formalin-fixed, paraffin-embedded (FFPE) slides (obtained from the UMCG Pathology Biobank) were deparaffinized and rehydrated in graded ethanol. Antigen retrieval was instigated by 15 minutes of microwave in pre-heated 10 mmol/L citrate buffer (pH 6.0). Endogenous peroxidase was blocked by incubating the slides in 0.45% hydrogen peroxide solution for 30 minutes. To stain for STING and CD8, the slides were incubated overnight at 4°C with, respectively, monoclonal rabbit anti-human TMEM173 antibody (EPR13130, ab181125, Abcam) and monoclonal mouse anti-human CD8 antibody (clone C8/144B, Agilent [Dako], M710301), diluted 50X in PBS containing 1% BSA and 1% human AB serum. Next, the slides were incubated for 30 minutes with Envision+/HRP anti-rabbit or anti-mouse antibody (Agilent [Dako]). Signal was visualized with 3,3'-diaminobenzidine (DAB) solution containing hydrogen peroxide, and the slides were counterstained with hematoxylin. In between incubations, the slides were washed with PBS. The sections were dehydrated and embedded in Eukitt quick-hardening mounting medium (Sigma Aldrich), and the slides were scanned using a Hamamatsu digital slide scanner (Hamamatsu Photonics). The numbers of STING + and $CD8^+$ cells in each slide were quantified automatically using QuPath v0.1.2 image analysis software[27] after manual selection of tumor epithelium sections.

2.4 | Statistics

Genotype counts were compared between patients and controls and the effects of allelic variants on clinical characteristics were assessed using chi-square testing. The effect of allelic variants on the risk of developing cervical cancer was determined by comparing allele frequencies between patients and controls and patients and the European reference cohort using odds ratios and confidence intervals, not adjusted for any external variable. Differences between age of diagnosis, *STING1* expression levels (delta Ct values), STING levels, and CD8 infiltration (number of positive cells per mm^2) between the study groups were analyzed using Kruskal-Wallis testing with post-Dunn tests. Survival was analyzed with Kaplan-Meier curves (log-rank) and univariate and multivariate Cox regression tests. Variables with a *P*-value < .05 in the univariate analyses were included in the multivariate analyses (Forward Stepwise LR). Significant associations were defined by a *P*-value lower than 0.05. Statistics were performed using SPSS software version 23.0 (IBM SPSS Statistics) or GraphPad Prism 7.02 (GraphPad Software).

3 | RESULTS

3.1 | Allelic variants in STING1 are not enriched in cervical cancer

We speculated that allelic variants in the cGAS-STING pathway could predispose to the development of cervical cancer due to the failure of the innate immune system to clear HPV-infected cells. To investigate this hypothesis, we genotyped *STING1* (encoding STING) using cervical scrapings of 150 cervical cancer patients and 20 age-matched healthy controls and compared the results with 503 individuals in the European reference cohort.[26] In the patient cohort, we found the following genotypes: WT/WT (58.0%), WT/HAQ (18.0%), WT/R232H (14.7%), WT/AQ (0.7%), HAQ/HAQ (3.3%), HAQ/R232H (2.0%), R232H/R232H (2.7%), and R232H/AQ (0.7%) (Table 1). Only four of these eight genotypes were found in the healthy controls: WT/WT (55.0%), WT/HAQ (15.0%), WT/R232H (20.0%), and HAQ/R232H (5.0%). In addition, one healthy control had the genotype WT/A313T (5.0%), which was not observed in the patient cohort.

Notably, the well-described HAQ variant was not found as homozygous genotype in the healthy control cohort. The genotypes WT/G230A, HAQ/AQ, and AQ/AQ, which have been reported for the European reference cohort,[26] were not found in both the patient and healthy control cohorts. However, the overall distribution of *STING1* genotypes within the patient population was comparable to the distribution of the healthy controls and European reference cohort. About half (58.0%) of the patient samples were characterized by two wild-type STING alleles (WT/WT), lacking each of the examined variants, whereas the minority of patients contained a single allele (WT/VAR, 33.4%) or two variant alleles (VAR/VAR, 8.7%), containing at least one of the examined substitutions on one allele or both alleles, respectively. This genotype distribution was comparable to the distribution found within the group of healthy controls (55.0%, 40.0%, and 5.0%, respectively) and within the European reference population (51.3%, 38.4%, and 10.4%, respectively).[26] In addition, the prevalence of individual *STING1* alleles in the cohort of cervical cancer patients was similar as well, with no allele showing an (significant) enrichment within the patient cohort (Table S1). The synonymous substitution rs7447927 (V48V) had a slightly, but not significantly, higher abundance in the European reference cohort than in the (Dutch) patient and healthy control cohorts (patient vs. European reference cohort $P = .074$). Altogether, we suggest that allelic variants of *STING1* are not a crucial factor for persistent infection of HPV and that these variants do not predispose to the development of cervical cancer.

3.2 | Homozygous variants of STING1 are associated with cervical adenocarcinomas

We next assessed whether the allelic variants could affect disease progression by analyzing the clinical characteristics of the patients (Table 2, Table S2). As to tumor typing, 59.8% of the patients with WT/WT genotype had squamous cell carcinomas and 35.6% had adenocarcinomas (Figure S1A). In contrast, we found that only 23.1% of patients with VAR/VAR genotype had squamous cell carcinomas, whereas nearly 70% of these patients presented with adenocarcinomas. Patients with WT/VAR genotypes showed histological distribution comparable to patients with a WT/WT genotype. Although allelic variants appear to skew towards adenocarcinoma subtype, the overall difference in histological distribution between the three groups WT/WT, WT/VAR, and VAR/VAR did not reach significance ($P = .138$).

We also noticed that the tumor diameters of patients with WT/WT and WT/VAR genotypes tended to be larger than those of patients with a VAR/VAR genotype (Figure S1B). In addition, nearly 70% of patients with VAR/VAR genotypes were diagnosed with early-stage cervical cancer (up to FIGO stage IB1) with a highest FIGO classification of IIB (Figure S1C). In contrast, only half of the patients with at least one wild-type allele were diagnosed with early-stage cervical cancer, and the rest was diagnosed with FIGO stage IB2 or higher. Here, the highest FIGO stage found was IVB.

TABLE 2 Clinical characteristics of patients divided in *STING1* genotype groups, based on monoallelic or biallelic occurrence of variants

	Biallelic wild type	Monoallelic variant	Biallelic variant
Patients	87 (58.0)	50 (33.3)	13 (8.7)
Age at diagnosis (in years)			
Median	51.3	48.2	40.1
Range	22.9-83.9	27.3-87.9	25.2-58.3
HPV status			
Negative	3	4	0
Positive	34	9	2
Unknown	50	37	11
FIGO stage			
IA	1 (1.1)	1 (2.0)	0 (0.0)
IB1	39 (44.8)	26 (52.0)	9 (69.2)
IB2	10 (11.5)	3 (6.0)	1 (7.7)
IIA	13 (14.9)	6 (12.0)	1 (7.7)
IIB	15 (17.2)	10 (20.0)	2 (15.4)
IIIA	1 (1.1)	0 (0.0)	0 (0.0)
IIIB	6 (6.9)	2 (4.0)	0 (0.0)
IVA	1 (1.1)	1 (2.0)	0 (0.0)
IVB	1 (1.1)	1 (2.0)	0 (0.0)
Histology			
Squamous cell carcinoma	52 (59.8)	28 (56.0)	3 (23.1)
Adenocarcinoma	31 (35.6)	21 (42.0)	9 (69.2)
Other	4 (4.6)	1 (2.0)	1 (7.7)
Grade of differentiation			
Good/moderate	40 (46.0)	25 (50.0)	7 (53.8)
Poor/undifferentiated	29 (33.3)	16 (32.0)	5 (38.5)
Unknown	18 (20.7)	9 (18.0)	1 (7.7)
Lymphangioinvasion			
No	60 (69.0)	30 (60.0)	8 (61.5)
Yes	26 (29.9)	20 (40.0)	5 (38.5)
Unknown	1 (1.1)	0 (0.0)	0 (0.0)
Tumor diameter (cm)			
0-4	42 (48.3)	21 (42.0)	9 (69.2)
≥4	27 (31.0)	17 (34.0)	2 (15.4)
Unknown	18 (20.7)	12 (24.0)	2 (15.4)
Primary treatment			
Wertheim-Meigs	46 (52.9)	29 (58.0)	9 (69.2)
Radio-chemotherapy	32 (36.8)	14 (28.0)	2 (15.4)
Other	9 (10.3)	7 (14.0)	2 (15.4)
Follow-up (in years)			
Median	4.63	4.60	3.79
Range	0.08-10.81	0.04-11.72	0.22-7.01

(Continues)

TABLE 2 (Continued)

	Biallelic wild type	Monoallelic variant	Biallelic variant
Results last follow-up			
No evidence of disease	63 (72.4)	36 (72.0)	6 (46.2)
Evidence of disease	1 (1.1)	1 (2.0)	0 (0.0)
Death of disease	17 (19.5)	13 (26.0)	7 (53.8)
Death of other disease	6 (6.9)	0 (0.0)	0 (0.0)

Abbreviation: HPV, human papillomavirus.

Patients with VAR/VAR genotypes tend to receive surgery more often than radiotherapy as their primary treatment (Figure S1D), which may be explained by their smaller tumor sizes and lower FIGO stages.

However, altogether, our analyses showed no significant difference between any of the discussed clinical factors.

3.3 | Homozygous variants of STING1 are prognostic factors in cervical cancer

Previous reports show that the 5-year survival of cervical cancer patients with metastasis is 16.5% compared with 91.5% without metastasis.[28] In addition, in cancer models of chromosomal instability, metastasis was promoted in a STING-dependent manner.[7] Therefore, we assessed whether particular allelic variants of *STING1* were associated with metastasis. As expected, the occurrence of metastasis was significantly associated with poor outcome (Table 3, univariate cox regression, $P < .001$). However, we observed that the proportion of patients that presented with distant and lymph node metastasis was comparable for each of the WT/WT, WT/VAR, and VAR/VAR groups (Figures S1E and F, respectively), indicating that the occurrence of metastasis is independent of the different *STING1* variants. Although distant metastasis was significantly associated with a poor prognosis in the group of patients with at least one wild-type allele (Kaplan-Meier, $P < .001$, not shown), we observed that occurrence of metastasis did not affect the outcome of patients with VAR/VAR genotypes of STING (Kaplan-Meier, $P = .382$, not shown). These patients have a poor prognosis regardless of having metastasis or not, indicating that another factor causes the poor outcome of these patients.

To follow up on these findings, we assessed the effect of *STING1* variants on the disease-specific survival (DSS). Interestingly, the DSS of patients with HAQ/HAQ, R232H/R232H, and R232H/AQ genotypes was significantly worse than with WT/WT genotype ($P = .049$, $P = .022$, and $P = .001$, respectively, not shown). As the individual genotype groups were rather small, we next compared the DSS between grouped WT/WT, WT/VAR, and VAR/VAR genotypes and observed a significant difference in outcome, with patients with homozygous allelic variants of *STING1* having a worse DSS than patients with wild-type or heterozygous variants (Figure 1A, $P = .019$). Although the DSS was slightly worse for the WT/VAR patients than for the WT/WT patients, this difference was not statistically significant ($P = .114$). Next, we hypothesized that the presence of at least one wild-type allele may result in functional STING and therefore grouped the patients with at least one wild-type allele for additional analysis of DSS. We show that having at least one wild-type allele significantly improves overall survival and DSS (Figure 1B, $P = .007$). Importantly, multivariate analysis of *STING1* genotype groups and clinicopathological factors revealed an independent association of *STING1* status with DSS (Table 3). Analysis of *STING1* expression levels by qRT-PCR showed that the prognostic value was also independent of *STING1* mRNA levels, as these were statistically comparable for WT/WT, WT/VAR, and VAR/VAR genotypes (Figure S2A, $P = .207$). Moreover, there was no significant difference in DSS (Figure S2B, $P = .617$) or in recurrence-free survival (Figure S2C, $P = .226$) based on above- and below-median *STING1* mRNA levels. Similar results were found when immunohistochemically assessing STING protein levels in paraffin-embedded tumor tissue (Figure S3). We hypothesized that STING variants may affect responsiveness to therapy. Indeed, the DSS was worse for patients with homozygous STING variants, independently of the primary treatment being surgery or radiochemotherapy (RCT) (Figure 1F-1I). These data demonstrate that homozygous mutated variants of *STING1* have independent prognostic value for patients with cervical cancer that cannot be explained by the levels of both STING mRNA and protein.

3.4 | Homozygous mutated variants of STING1 appear to be associated with early onset of cervical cancer

To understand the worse survival of VAR/VAR STING patients, we further investigated differences in the clinical data of the patients. We noticed that the age of diagnosis significantly differed between the three genotype groups (Figure 1C, $P = .0395$). Specifically, the age of diagnosis was significantly lower for patients with VAR/VAR genotypes of *STING1* than for patients with WT/WT ($P = .0331$). The median ages of diagnosis were 40.1 vs 51.3 and 48.2 years, respectively (Table 2). Especially the HAQ/HAQ (39.6 years) and R232H/R232H (36.9 years) genotypes contributed to this lower median age. When we subsequently compared the effect of the age at the time of diagnosis on the outcome, we observed that the DSS within the entire cohort of 150 patients was not significantly different between younger and older age of diagnosis (Figure S4, based on the median age of the entire cohort of 50.05, $P = .224$). This was also not the case when first stratifying the cohorts for adenocarcinoma vs squamous cell carcinoma ($P = .146$ vs $P = .822$, not shown). Thus, early onset of disease in itself is not a predictor of poor prognosis. Therefore, we suggest an independent negative effect of homozygous variants of *STING1* on the survival of cervical cancer patients.

TABLE 3 Uni- and multivariate Cox regression survival analyses based on clinical and *STING1* parameters

	Univariate				Multivariate[a]				Multivariate[b]			
	HR	95% CI		*P*-value	HR	95% CI		*P*-value	HR	95% CI		*P*-value
Age of diagnosis (cont)	0.977	0.954	1002	.070								
Age (in years)												
<50.04 median	ref	ref	ref	ref								
>50.04 median	0.657	0.333	1.298	.227								
Grade												
Good	ref	ref	ref	ref								
Average	0.980	0.358	2.685	.969								
Bad/not	1.204	0.466	3.109	.701								
Unknown	1.341	0.456	3.944	.594								
FIGO stage												
I	ref	ref	ref	ref								
II	3.187	1.445	7.031	.004								
III	6.959	2.374	20.401	<.001								
IV	14.684	4.493	47.988	<.001								
Typing												
Squamous	ref	ref	ref	ref								
Adeno	1.148	0.583	2.260	.690								
Other	0.872	0.116	6.542	.894								
Distant metastasis												
No	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
Yes	14.952	7.160	31.225	<.001	14.458	6.327	33.041	<.001	14.970	6.410	34.963	<.001
Lymph node metastasis												
No	ref	ref	ref	ref								
Yes	8.637	3.762	19.830	<.001								
Tumor diameter (cm)												
0-4	ref	ref	ref	ref								
≥4	1.655	0.808	3.389	.168								
Unknown	0.771	0.297	2.002	.594								
Primary treatment												
WM	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref	ref
RCT	3.753	1.618	8.705	.002	3.005	1.226	7.368	.016	2.860	1.176	6.956	.020
Other	10.413	4.143	26.169	<.001	12.163	4.317	34.269	<.001	11.559	4.152	32.175	<.001
STING1 genotype												
WT/WT	ref	ref	ref	ref	ref	ref	ref	ref				
WT/VAR	1.399	0.672	2.912	.369	2.169	0.999	4.708	.050				
VAR/VAR	3.393	1.385	8.310	.008	3.516	1.315	9.397	.012				
V48V/V48V												
WT/WT	ref	ref	ref	ref					ref	ref	ref	ref
WT/VAR	1.271	0.600	2.689	.531					2.251	1.007	5.032	.048
VAR/VAR	3.564	1.512	8.400	.004					2.930	1.146	7.497	.025
CD8 infiltrate (cont)	1.000	0.999	1.000	.458								

(Continues)

TABLE 3 (Continued)

	Univariate				Multivariate[a]				Multivariate[b]			
	HR	95% CI		*P*-value	HR	95% CI		*P*-value	HR	95% CI		*P*-value
CD8 infiltrate[c]												
Low	ref	ref	ref	ref								
High	0.846	0.363	1.972	.699								

Note: Disease-specific survival, Cox regression - Enter for univariate and Forward Stepwise (LR) for multivariate, n = 150.

Abbreviations: CI: Confidence Interval; FIGO: International Federation of Gynecology and Obstetrics; HR: Hazard Ratio; RCT: Radiochemotherapy.

[a]When STING1 genotype entered as variable, V48V/V48V not included.

[b]When V48V/V48V entered as variable, STING1 genotype not included.

[c]Based on below- or above-median CD8 count of 392 125 per mm^2.

3.5 | Homozygous variants of STING1 are associated with higher frequency of recurrences

In addition to being diagnosed at an earlier age, patients with VAR/VAR variants notably suffered from recurrent disease significantly more often than patients with the WT/WT variant (Figure 1D, *P* = .018). To confirm whether the occurrence of recurrent disease explains the worse outcome of patients with VAR/VAR *STING1*, we performed additional Kaplan-Meier analyses in which we excluded either all patients with recurrences or only the patients with VAR/VAR genotypes with recurrences. In both cases, there was no difference in survival between the groups (*P* = .942 and *P* = .642, respectively, not shown). In accordance, the recurrence-free survival was significantly better for the WT/WT group of patients (Figure 1E, *P* = .013) and, although insignificantly, for the WT/VAR group (*P* = .061), compared with the VAR/VAR group of patients. In total, 27 out of 150 patients suffered from a recurrence. From the 21 patients within this group with at least one WT allele, six (28.6%) survived the recurrent disease, whereas the six VAR/VAR patients that recurred all succumbed to the disease (not shown). Altogether, these findings show that although the initial clinical characteristics such as tumor diameter and FIGO stage of patients with VAR/VAR genotypes of STING appear slightly beneficial over patients with WT/WT and WT/VAR, having at least one wild-type allele may protect against (aggressive) recurrent disease and therefore improve outcome.

3.6 | The prognostic value of mutated STING1 variants is independent of $CD8^+$ T cell infiltration

High levels of STING protein expression were previously associated with high $CD8^+$ T cell infiltration.[17] In line with this, knockout of STING led to decreased infiltration of $CD8^+$T cells in animal models.[8] Although we observed no differences in STING expression between the genotypes, we hypothesized that the patients with VAR/VAR genotypes of *STING1* might have lower $CD8^+$T cell infiltration, possibly explaining the poor survival of these patients. To investigate this, we performed immunohistochemistry for $CD8^+$T cells using paraffin-embedded tumor tissue of 99 cervical cancer patients from our cohort. Quantification of $CD8^+$ T cells across the FFPE slides was performed using machine-based quantification (Figure 2A). We found that $CD8^+$ T cell infiltration did not differ between the three *STING1* groups (Figure 2B, *P* = .687). Patient outcome in this cohort also did not reach statistical significance for outcome based on low and high $CD8^+$ T cell infiltration (Figure 2C and Table 3, *P* = .699). Lastly, there was no association between $CD8^+$ T cell infiltration and *STING1* expression (*P* = .295, not shown). Thus, homozygous variants of STING are prognostic factors in cervical cancer, independently of both *STING1* expression and $CD8^+$ T cell infiltration.

3.7 | The homozygous V48V variant of STING1 represents a surrogate genetic marker for STING1 variations associated with poor outcome in cervical cancer

When assessing the effect of having two wild-type alleles, one wild-type allele, or no wild-type allele of each individual nucleotide substitution on survival, we found that most individual substitutions did not significantly affect DSS (not shown). Only the 14 patients with homozygous V48V presented with significantly worse outcome than patients with homozygous or heterozygous wild-type V48V (Figure 3A, *P* = .008). In addition, we observed that half of these patients suffered from recurrent disease, in contrast to patients with WT/WT and WT/VAR genotypes (14.0% and 16.0%, respectively) (Figure 3B, *P* = .005). In accordance, the recurrence-free survival was significantly worse for patients with homozygous mutated V48V when comparing the patients only based on this single-nucleotide substitution (Figure 3C, *P* = .005). Interestingly, V48V represents a synonymous substitution and therefore likely has no clinical implications in itself. Moreover, we described earlier that V48V variant is not enriched in the patient cohort. However, homozygous presence of V48V almost completely corresponded to the patients with VAR/VAR genotypes of STING, with 13 patients having R232H/R232H, HAQ/HAQ, HAQ/R232H, or R232H/AQ (VAR/VAR) genotypes and only one of the 14 patients with homozygous V48V having a WT/AQ (WT/VAR) genotype of *STING1*. Altogether, our findings therefore indicate that homozygous V48V in *STING1* may potentially be used

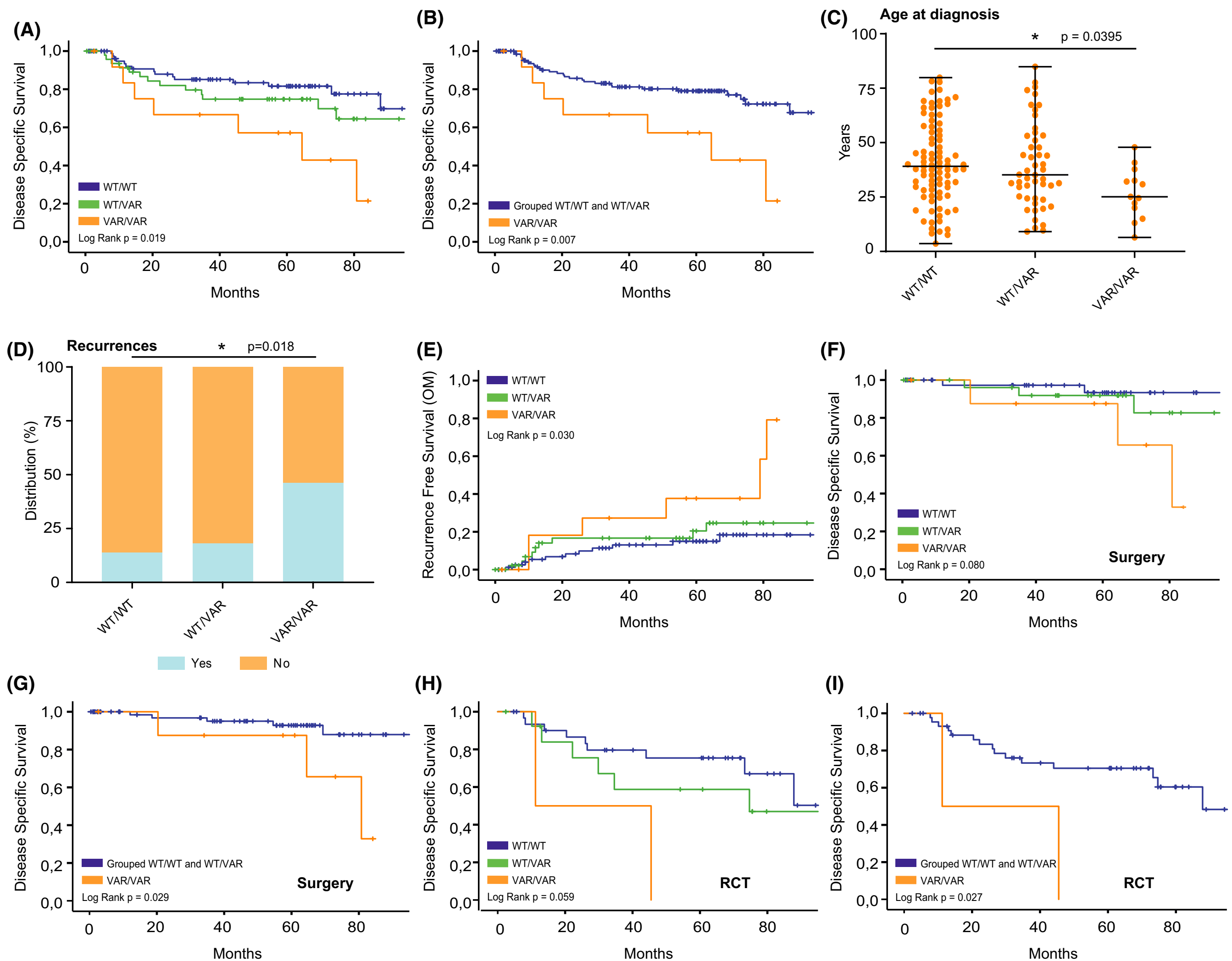

FIGURE 1 Cervical cancer patients were analyzed regarding disease-specific survival (A, B), based on three groups (A): WT/WT (blue), WT/VAR (green), and VAR/VA (orange) or on two groups (B): WT/WT and WT/VAR combined (blue) and VAR/VAR (orange). C, Age at the time of diagnosis (in years) for the three described groups. Each orange dot represents one patient. Statistical analysis was performed by one-way ANOVA with post-hoc Kruskal-Wallis test. D, Distribution of recurrences (%) among the three described groups: Yes (recurrence, light blue) or No (no recurrence, orange). Statistical analysis was performed by Pearson's chi-square testing. E, Recurrence-free survival (one-minus plot) for WT/WT (blue), WT/VAR (green), and VAR/VAR (orange). F-I, Disease-specific survival distinguishing cervical cancer patients by primary treatment being surgery (F, G,) or RCT (H, I) and by distinguishing three groups (F, H): WT/WT (blue), WT/VAR (green), and VAR/VA (orange) or two groups (G, I): WT/WT and WT/VAR combined (blue) and VAR/VAR (orange). For all survival curves, statistical analyses were performed by log-rank testing. Significance was defined as $P < .05$. Curves were cut off at 7.5 years

as a surrogate marker for a poor *STING1* genotype–related outcome in cervical cancer.

4 | DISCUSSION

In this study, we genotyped the STING-encoding *STING1* gene across a unique cohort of 150 cervical cancer patients and examined a panel of clinical characteristics. Notably, we found that homozygous variants of *STING1* were significantly associated with worse survival outcome. This association was found to be independent of $CD8^+$ T cell infiltration and *STING1* expression. Thus, specific allelic variants of *STING1* may affect the development and progression of cervical cancer.

STING-encoding *STING1* is one of the genes described to influence the susceptibility of an individual to cervical cancer.[3] We genotyped regions in the STING-encoding *STING1* that can contain key single-nucleotide substitutions using cervical scrapings of 150 cervical cancer patients to investigate whether particular allelic variants in *STING1* are enriched in cervical cancer patients and have a prognostic value. In accordance with the previous conclusion of Xiao et al that no variant of *STING1* is associated with the risk of cervical precancerous lesions,[29] we found no allelic variant of *STING1* to be enriched in our cervical cancer patient cohort compared with healthy controls and a European reference population. This suggests that these allelic variants are not a crucial factor for persistent infection with HPV and that they do not predispose to the development of cervical cancer.

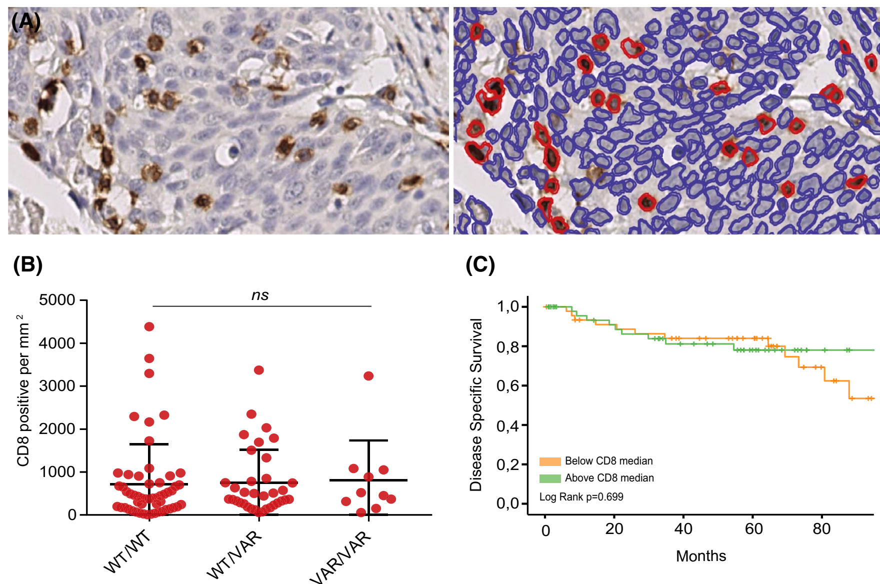

FIGURE 2 Immunohistochemical staining of CD8 on formalin-fixed, paraffin-embedded (FFPE) slides of tumors obtained from the studied cervical cancer patient cohort. A, Microscopic image (400X magnification) of CD8 staining (left) and representation of the computerized quantification of membrane 3,3'-diaminobenzidine (DAB)-positive cells (red) and DAB-negative cells (blue) in QuPath image analysis software (right). B, Quantified numbers of DAB/CD8-positive cells per mm^2 tumor tissue for the three groups: WT/WT, WT/VAR, and VAR/VAR. C, Disease-specific survival dividing patients into two groups: below (orange) and above (green) the median number of CD8 infiltrating cells. Statistical analysis was performed by log-rank testing. Significance was defined as $P < .05$. Curve was cut off at 7.5 years

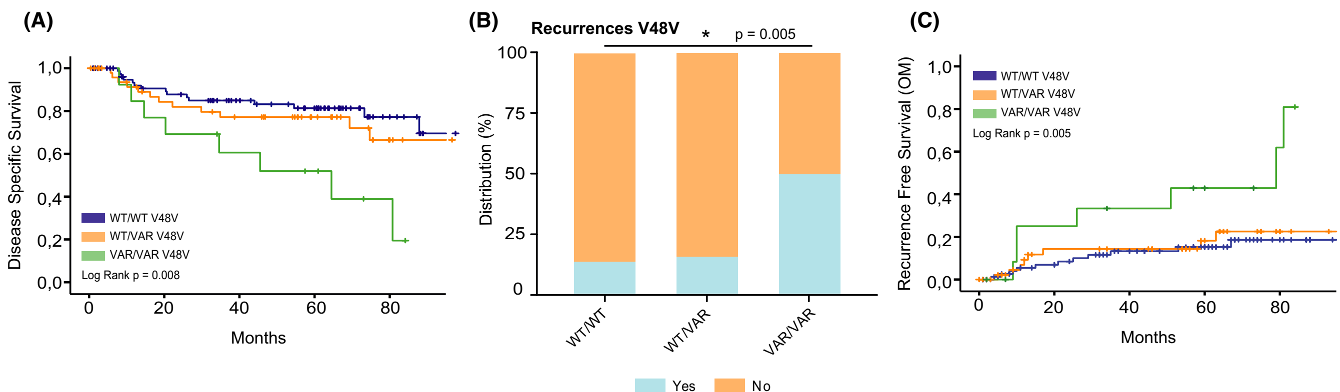

FIGURE 3 Analysis of the single synonymous substitution rs7447927 V48V. A, Disease-specific survival dividing patients into three groups: WT/WT (blue), WT/VAR (orange), and VAR/VAR (green) ($P = .008$). B, Distribution of recurrences (%) among the three described V48V groups: Yes (recurrence, light blue) or No (no recurrence, orange). Statistical analysis was performed by Pearson's chi-square testing. C, Recurrence-free survival (one-minus plot) based on the three described V48V groups. Statistical analyses for survival curves were performed by log-rank testing. Significance was defined as $P < .05$. Curve was cut off at 7.5 years

Typically, the predominant histological subtype of cervical cancer is squamous cell carcinoma (70%-80% of cases), followed by adenocarcinoma (10%).[2] Interestingly, we found that the nearly 70% of patients in our cohort with VAR/VAR *STING1* genotypes presented with adenocarcinomas. Moreover, we noticed that the tumor diameters of patients with WT/WT and WT/VAR genotypes often were larger, and the FIGO stages at diagnoses were often higher than those of patients with VAR/VAR genotypes, although these differences did not reach statistical significance. In addition, we observed that patients with VAR/VAR genotypes of *STING1* were diagnosed with cervical cancer at a significantly earlier age. The median age at diagnosis of cervical cancer is 48 years.[30] In accordance, we found that the median ages of WT/WT and WT/VAR patients were 51.3 and 48.2, respectively. In contrast, the median age of diagnosis for patients with homozygous variants was 40.1 years. About half of the patients that were diagnosed at an age below the median of the entire cohort presented with adenocarcinomas. These findings are in accordance with previous reports that patients with adenocarcinomas are generally diagnosed at an earlier stage and at a younger age.[31] Together, our findings may indicate again a link between homozygous variants of *STING1*, histology, and age of diagnosis.

Women with locally advanced cervical cancer have a higher rate of local and distant recurrences and worse survival than women that

are diagnosed with early-stage disease.[32,33] Interestingly, despite their tendency toward having early-stage disease, patients with VAR/VAR genotypes of STING had significantly worse disease-specific and recurrence-free survival than patients with WT/WT and WT/VAR genotypes. Hence, having at least one wild-type allele may partially protect against (aggressive) recurrent disease and thereby improve outcome. As reported before,[30] the outcome of the patients was independent of age at the time of diagnosis. It was previously observed in multiple cancer types and models that decreased levels of STING lead to poor induction of IFN-I.[10,12-16,34] To possibly explain the worse survival of patients with homozygous variants of STING, we assessed the mRNA and protein levels of STING. However, we did not observe lower expression of *STING1* and STING in the patients with homozygous variants as compared with patients with at least one wild-type allele. Moreover, survival was comparable for high vs low levels of both. After 60 months of follow-up, patients with *STING1* mRNA expression above median appear to gain a slight survival advantage. We determined mRNA levels using cervical scrapings and lack material to determine potential change in mRNA levels during follow-up time that may explain this advantage. Possibly, a higher mRNA level at baseline may provide a long-term survival advantage. However, based on our data, we cannot draw any conclusions on this.

Despite apparent intact translation, STING variants may be dysfunctional. For example, it was described that the R232H, G230A, and R293Q substitutions are located in the cyclic dinucleotide (CDN)-binding region of the gene and cause defective response of STING.[23] A recent study regarding the effect of the homozygous HAQ variant of STING in individuals infected with HIV showed that this variant contributes to reduced levels of IFN production and a reduced immune response.[24] In accordance, it is reported that this variant is associated with defective functionality of STING due to the occurrence of the rs13181561 substitution upstream of *STING1* in individuals with HAQ/HAQ variants of *STING1*.[19,20] We did not look into HPV proteins, nor did we assess the presence of the rs13181561 substitution and other substitutions such as the synonymous rs7447927[35] that has been reported previously with regard to other cancer types. In addition, the limited availability of cDNA did not allow us to study the IFN-I mRNA levels.

As IFN-I signaling ultimately leads to infiltration of CD8 T cells, we performed immunohistochemistry for CD8 on available tumor tissue. We observed no difference in $CD8^+$ T cell numbers between the patient groups with different *STING1* variants, indicating intact IFN signaling in patients with hetero- and homozygous variants of STING. However, it was formerly reported that HPV+ human head and neck squamous cell carcinomas present with less clonal expansion of cytotoxic T cells and lower levels of antigen-presenting machinery than carcinomas lacking HPV.[36] As cervical cancers are almost exclusively HPV+, it is possible that although patients with homozygous variants of STING have similar numbers of $CD8^+$-infiltrated cells compared with patients with at least one wild-type allele, these CD8 infiltrates may merely represent irrelevant “bystander” CD8 cells that do not effectuate actual antitumor immunity. In line, Fu et al showed in mice that activation of dendritic cells was associated with STING-dependent phosphorylation of IRF3, and that antitumor efficacy upon treatment with a CDN-based vaccine depended on STING and $CD8^+$ T cells.[37] Moreover, immune checkpoint inhibition did not induce tumor regression in the context of STING loss,[8] suggesting a lack of tumor-reactive $CD8^+$ T cells. Studies are currently ongoing to induce STING-mediated immunity, either directly via eg STING agonists[18] or indirectly through radiotherapy or inhibition of the DNA damage repair pathway.[38] As we speculate that homozygous variants of STING are associated with impaired antitumor immunity and fail to induce the activation of tumor-reactive $CD8^+$ T cells, we hypothesize that STING-inducing therapy may not be effective in patients with these variants. Therefore, we recommend that these studies should include the effect of various *STING1* variants on (immune)therapeutic response against cervical cancer.

Additionally, treatment regimens for cervical cancer are primarily determined based on FIGO stage. Early-stage cancer is usually treated with surgery, whereas for late-stage disease, patients can be treated with primary or palliative (chemo)radiation therapy or a combination treatment.[32] In accordance with their large proportion of early FIGO stages and small tumors, patients with VAR/VAR genotypes of STING are mainly treated with surgery. However, we demonstrated that these patients have a poor outcome and observed a survival disadvantage even in the group of patients that were primarily treated with RCT. Therefore, we speculate the treatment regimen for patients with homozygous variants of STING may be ineffective and potentially should be reconsidered.

Finally, although V48V was previously associated with esophageal squamous cell carcinoma in Chinese individuals,[35] here it appeared to be more abundant in the European reference cohort than in the (Dutch) patient and healthy control cohorts (patient vs European reference cohort $P = .074$). Interestingly, despite representing a synonymous substitution, V48V was previously reported to be in linkage disequilibrium with both HAQ and R232H and the rs13181561 substitution, making it a surrogate marker for loss of STING function.[35] Here, we showed that the V48V indeed corresponded to homozygous variants of *STING1* and that it was individually prognostic for outcome. As the allelic variants represent germline and not somatic substitutions specific for the cervical tissue, it is possible to identify V48V in the DNA of patients, which may facilitate genotyping for prognostic purposes.

Altogether, our results suggest that patients with homozygous allelic variants of the *STING1* gene have worse DSS and recurrence-free survival and earlier age of diagnosis than patients with at least one wild-type *STING1* allele. Homozygous V48V was found to be individually prognostic and may be investigated as a novel, surrogate prognostic biomarker in cervical cancer.

DISCLOSURE

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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