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 antitumor 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

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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. A persistent human papillomavirus (HPV) infection underlies the development of cervical cancer. In recent years, several genomewide association studies have led to the discovery of multiple genes influencing the susceptibility to cervical cancer. Allelic variants of these genes together may explain up to 24% of the variance in the risk for developing cervical cancer. 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.

In the context of cancer, the cGAS-STING pathway can identify chromosomal instability in cancerous cells by detecting cytoplasmic DNA or identify infection with potentially carcinogenic viruses by detecting viral DNA, such as DNA from HPV. In addition, it is reported to improve the efficacy of (DNA damage–inducing) radiotherapy. 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 in multiple cancer types such as breast, gastric, and hepatocellular carcinoma, levels of STING were found to be decreased compared with healthy tissue. In another study regarding DNA damage, loss of STING hampered tumor regression upon application of multiple therapeutic strategies such as immune checkpoint blockade. As loss and decreased levels of STING are associated with poor prognosis, the potential therapeutic benefit of inducing STING is currently under investigation. 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. 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). 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). In combination, the latter three are termed the HAQ genotype, which occurs in 20.4% of the human population. The R232H, G230A, and R293Q substitutions are located in the CDN-binding region of the gene and cause defective response of STING. 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.

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 uterine cervical 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. To determine prevalence of allelic variants, data from the NCBI 1000 Genomes phase 3 browser 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: V48F, 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%. Combinations of single-nucleotide substitutions were annotated either as HAQ genotype (V48F, R71H, G230A, and R293Q), R232H genotype (V48F and R232H), or AQ genotype (V48F, G230A,
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 preheated 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 software27 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 mm2) 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/AQ (1.0%) (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.

| Genotype   | 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)                 | -                         |
| 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)                   |

TABLE 1 Distribution of STING1 genotypes across patient and control cohorts

and R293Q); A313T 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, 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). 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  | 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) |               |                     |                   |
| ≤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)
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.\(^2\) 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 versus 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 2** (Continued)

| Results last follow-up | Biallelic wild type | Monoallelic variant | Biallelic variant |
|------------------------|---------------------|---------------------|------------------|
| 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.
| TABLE 3 Uni- and multivariate Cox regression survival analyses based on clinical and STING1 parameters |
|---------------------------------------------------------------|
| **Univariate** | **Multivariate** | **Multivariate** |
| **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 |
| Yes | 14.952 | 7.160 | 31.225 | <.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 |
| RCT | 3.753 | 1.618 | 8.705 | .002 |
| Other | 10.413 | 4.143 | 26.169 | <.001 |
| STING1 genotype WT/WT | ref | ref | ref | ref |
| WT/VAR | 1.399 | 0.672 | 2.912 | .369 |
| VAR/VAR | 3.393 | 1.385 | 8.310 | .008 |
| V48V/V48V WT/WT | ref | ref | ref | ref |
| WT/VAR | 1.271 | 0.600 | 2.689 | .531 |
| VAR/VAR | 3.564 | 1.512 | 8.400 | .004 |
| CD8 infiltrate (cont) | 1.000 | 0.999 | 1.000 | .458 |

(Continues)
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.\(^{27}\) In line with this, knock-out 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...
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 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.
Typically, the predominant histological subtype of cervical cancer is squamous cell carcinoma (70%-80% of cases), followed by adenocarcinoma (10%). 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. 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. 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.\textsuperscript{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,\textsuperscript{29} 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-1.\textsuperscript{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 functional. For example, it was described that the R232H, G230A, based on our data, we cannot draw any conclusions on this. 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.

As IFN-1 signaling ultimately leads to infiltration of CD8\textsuperscript{T} cells, we performed immunohistochemistry for CD8 on available tumor tissue. We observed no difference in CD8\textsuperscript{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.\textsuperscript{36} As cervical cancers are almost exclusively HPV+, it is possible that although patients with homozygous variants of STING have similar numbers of CD8\textsuperscript{T}-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\textsuperscript{T} cells.\textsuperscript{37} Moreover, immune checkpoint inhibition did not induce tumor regression in the context of STING loss,\textsuperscript{3} suggesting a lack of tumor-reactive CD8\textsuperscript{T} cells. Studies are currently ongoing to induce STING-mediated immunity, either directly via eg STING agonists\textsuperscript{18} or indirectly through radiotherapy or inhibition of the DNA damage repair pathway.\textsuperscript{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\textsuperscript{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.\textsuperscript{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,\textsuperscript{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.\textsuperscript{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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REFERENCES

1. Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Allen C, et al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-year, 1990 to 2015: a systematic analysis for the global burden of disease study. *JAMA Oncol*. 2017;3(4):524-548.

2. de Sanjose S, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Lancet Oncol*. 2010;11:1048-1056.

3. Chen D, Gyllensten U. Lessons and implications from association studies. *Trends Genet*. 2015;31:41-54.

4. Chen D, Cui T, EK WE, Liu H, Wang H, Gyllensten U. Analysis of the genetic architecture of susceptibility to cervical cancer indicates that common SNPs explain a large proportion of the heritability. *Carcinogenesis*. 2015;36:992-998.

5. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* (80.-). J. 2013;339:786-791.

6. Chung KW, Dhillon P, Huang S, et al. Mitochondrial damage and acetylation of the STING pathway lead to resistance to IL-12 and corticosteroids. *Cell Metab*. 2019;30:784-799.e5.

7. Bakhour SF, et al. Chromosomal instability drives metastasis through a cytosolic DNA response. *Nature*. 2018;553:467-472.

8. Harding SM, Ngo B, Laughney AM, et al. Mitotic progression following DNA damage enables pattern recognition within micrometastasis. *Nature*. 2017;548:466-470.

9. Li T, Chen ZJ. The cGAS-cGAMP-STING pathway connects DNA damage to inflammation, senescence, and cancer. *J Exp Med*. 2018;215(5):1287-1299.

10. Deng L, Liang H, Xu M, et al. STING-dependent cytosolic DNA sensing promotes radiation-induced type I interferon-dependent antitumor immunity in immunogenic tumors. *Immunity*. 2014;41:843-852.

11. McLaughlin MP, Patin EC, Pedersen M, et al. Inflammatory microenvironment remodelling by tumour cells after radiotherapy. *Nat Rev Cancer*. 2020;20(4):203-217.

12. Xia T, Konno H, Ahn J, Barber GN. Derepligeation of STING Signaling in Colorectal Carcinoma Constrains DNA Damage Responses and Correlates With Tumorigenesis. *Cell Rep*. 2016;14:282-297.

13. Xia T, Konno H, Barber GN. Recurrent loss of STING signaling in melanoma correlates with susceptibility to viral oncolysis. *Cancer Res*. 2016;76:6747-6759.

14. Bhatalia K, et al. Antiviral signaling protein MITA acts as a tumor suppressor in breast cancer by regulating NF-κB induced cell death. *Biochim Biophys Acta - Mol Basis Dis*. 2014;1842(2):144-153.

15. Song S, et al. Decreased expression of STING predicts poor prognosis in patients with gastric cancer. *Sci Rep*. 2017;7:1-13.

16. Bu Y, Liu F, Jia Q-A, Yu S-N. Decreased expression of TMEM173 predicts poor prognosis in patients with hepatocellular carcinoma. *PloS One*. 2016;11:e0165681.

17. Chon HJ, Kim H, Noh JH, et al. STING signaling is a potential immunotherapeutic target in colorectal cancer. *J Cancer*. 2019;10:4932-4938.

18. Corrales L, et al. Direct activation of STING in the tumor microenvironment leads to potent and systemic tumor regression and immunity. *Cell Rep*. 2015;11:1018-1030.

19. Patel S, et al. Response to comments on “The Common R71H–G230A–R293Q Human TMEM173 is a Null Allele”. *J Immunol*. 2017;198:4185-4188.

20. Patel S, Blauhoeber SM, Tucker HR, et al. (Baltimore, M, et al. 198(2): 776–787. 2017. doi:10.4049/jimmunol. 1601585. The common R71H–G230A–R293Q (HAQ) human TMEM173 is a null allele. *J Immunol*. 1950;198:776-787.

21. Yi G, Brendel VP, Shu C, et al. Single nucleotide polymorphisms of human STING can affect innate immune response to cyclic dinucleotides. *PloS One*. 2013;8:1-16.

22. Jin L, et al. Identification and characterization of a loss-of-function human MPYS variant. *Genes Immun*. 2011;12:263-269.

23. Patel S, Jin L. TMEM173 variants and potential importance to human biology and disease. *Genes Immun*. 2019;20(1):82-89.

24. Nissen SK, et al. Multiple homozygous variants in the STING-encoding TMEM173 gene in HIV long-term nonprogressors. *J Immunol*. 2018;200(10):3372-3382.

25. Gene [Internet]. Gene ID: 340061. *Homo sapiens STING (TP7.3.1) transmembrane protein 1*. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information, 1988.

26. The 1000 Genomes Project Consortium. A global reference for human genetic variation.*Nature*. 2015;526(7571):68-74.

27. Bankhead P, Loughrey MB, Fernández JA, et al. QuPath: Open source software for digital pathology image analysis. *Sci Rep*. 2017;7(1):16878.

28. Li H, Xiaohua Wu, Cheng X. Advances in diagnosis and treatment of metastatic cervical cancer. *Gynecol. Oncol*. 2016;27(4):e43.

29. Xiao D, Huang W, Ou M, et al. Interaction between susceptibility loci in cGAS-STING pathway, MHC gene and HPV infection on the risk of cervical precancerous lesions in Chinese population. *Oncotarget*. 2017;8:84228-84238.

30. Gao Y, Ma J, et al. The evaluation of older patients with cervical cancer. *Clin Interv Aging*. 2013;8:783-788.

31. Anton-Culver H, et al. Comparison of adenocarcinoma and squamous cell carcinoma of the uterine cervix: a population-based epidemiologic study. *Am J Obstet Gynecol*. 1992;166:1507-1514.

32. Cohen PA, Jhingran A, Oknain Denny L. Cervical cancer. *Lancet*. 2019;393:169-182.

33. Manders DB, et al. Locally advanced cervical cancer: outcomes with variable adherence to treatment. *Am J Clin Oncol Cancer Clin Trials*. 2018;41:447-451.

34. Zhong B, Yang Y, Li S, et al. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity*. 2008;29:538-550.

35. Wu C, Wang Z, Song X. Joint analysis of three genome-wide association studies of esophageal squamous cell carcinoma in Chinese populations. *Nat Genet*. 2015;47(4):1173-1178.

36. Saloura V, et al. Characterization of the T-cell receptor repertoire and immune microenvironment in patients with locoregionally advanced squamous cell carcinoma of the head and neck. *Clin Cancer Res*. 2017;23:4897-4907.

37. Fu J, Kanne DB, Leong M, et al. STING agonist formulated cancer vaccines can cure established tumors resistant to PD-1 blockade. *Sci Transl Med*. 2015;7:283ra52.

38. Luo X, Donnelly CR, Gong W, Chen Q, Leil YL. HPV16 drives cancer immune escape via NLRX1-mediated degradation of STING. *J Clin Investig*. 2020;130(4):1635-1652.

SUPPORTING INFORMATION

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

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