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ORIGINAL ARTICLE

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Validation and functional follow-up of cervical cancer risk variants at the HLA locus

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Cervical cancer is the fourth most common cancer in females. Genome-wide association studies (GWASs) have proposed cervical cancer susceptibility variants at the *HLA* locus on chromosome 6p21. To corroborate these findings and investigate their functional impact in cervical tissues and cell lines, we genotyped nine variants from cervical cancer GWASs (rs17190106, rs535777, rs1056429, rs2763979, rs143954678, rs113937848, rs3117027, rs3130214, and rs9477610) in a German hospital-based series of 1122 invasive cervical cancers, 1408 dysplasias, and 1196 healthy controls. rs17190106, rs1056429 and rs143954678/rs113937848 associated with cervical malignancies overall, while rs17190106 and rs535777 associated specifically with invasive cancer (OR = 0.69, 95% CI = 0.55–0.86, $p = 0.001$) or adenocarcinomas (OR = 1.63, 95%CI = 1.17–2.27, $p = 0.004$), respectively. We tested these and one

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previously genotyped GWAS variant, rs9272117, for potential eQTL effects on 36 gene transcripts at the *HLA* locus in 280 cervical epithelial tissues. The strongest eQTL pairs were rs9272117 and *HLA-DRB6* ($p = 1.9$x10E-5), rs1056429 and *HLA-DRB5* ($p = 2.5$x10E-4), and rs535777 and *HLA-DRB1* ($p = 2.7$x10E-4). We also identified transcripts that were specifically upregulated (*DDX39B*, *HCP5*, *HLA-B*, *LTB*, *NFKBIL1*) or downregulated (*HLA-C*, *HLA-DPB2*) in HPV+ or HPV16+ samples. In comparison, treating cervical epithelial cells with proinflammatory cytokine γ-IFN led to a dose-dependent induction of *HCP5*, *HLA-B*, *HLA-C*, *HLA-DQB1*, *HLA-DRB1*, *HLA-DRB6*, and repression of *HSPA1L*. Taken together, these results identify relevant genes from both the MHC class I and II regions that are inflammation-responsive in cervical epithelium and associate with HPV (*HCP5*, *HLA-B*, *HLA-C*) and/or with genomic cervical cancer risk variants (*HLA-DRB1*, *HLA-DRB6*). They may thus constitute important contributors to the immune escape of precancerous cells after HPV-infection.

KEYWORDS

cervical malignancy, eQTL, HPV, IFNG, replication, SNP, susceptibility

1 | INTRODUCTION

Cancer of the uterine cervix is the third most frequent malignancy in females aged 15–44 years in Germany with more than 4500 cervical cancer cases diagnosed overall in 2022.[1] Human papillomavirus (HPV) infection with high risk (hr) types such as HPV16 and HPV18 is detected in most cases, however, the infection alone is not sufficient to drive the development of cancer. While additional factors still remain to be fully elucidated, it is clear that the immune response of the host plays a crucial role in disease progression.[2,3] There is a known familial relative risk component to invasive cervical cancer, and previous studies have calculated heritability estimates between 27% and 36%.[4–6] Genome-wide association studies (GWASs) attempting to capture the genetic variants contributing to cancer susceptibility have implicated variants largely at the *HLA* locus (6p21), apart from findings at the 2q13 (*PAX8*), 17q12 (*GSDMB*), and 5p15 (*CLPTM1L*) loci, among others.[7–11]

The *HLA* locus was the first consistent cervical cancer susceptibility locus and multiple population-specific as well as universal signals at this locus have been discovered.[7,8,10–17] However, complex linkage patterns at this locus have made it difficult to identify the causal variants underlying this association, and the target genes have remained elusive. Current knowledge indicates that the GWAS locus at 6p21 is made up of several distinct signals nearby several distinct genes such as rs9272143/rs9271898/rs9272050/rs9272245/rs35508382/rs9270747/rs9272117 (*HLA-DQA1*, *HLA-DRB1*),[7,9,10,14,18] rs2516448/rs2844511 (*MICA*),[7,14,18] rs3117027/rs3130196 (*HLA-DPA2*, *HLA-DPB2*),[7,14] rs4282438 (*HLA-DPB1*),[8] rs73730372/rs55986091/rs36214159 (*HLA-DQA1*, *HLA DQB1*),[10,11,14] rs2856437 (*PBX2*),[9] rs6938453 (*MICA*),[10] rs9266183 (*HLA-B*),[10] rs1053726/ rs9266766 (*HLA-B*, *HLA-S*, *MICA*),[9,11] rs17190106 (*MUC22*, *HCG22*),[9] rs114060326 (*MICB/MCCD1*),[9] rs2763979 (*HSPA1B*),[9] rs34563311 (*HLA-DRB1*),[9] and rs535777 (*HLA-DRB1*, *HLA-DQA1*).[9] There have been only a few fine-mapping studies so far to identify cervical cancer and hrHPV specific HLA-alleles.[6,11,14,19–21]

Here, we investigated nine SNPs at six recently reported GWAS signals rs17190106,[9] rs535777,[9] rs1053726,[9,11] rs2763979,[9] rs6938453,[10] and rs3117027/rs3130196.[7,14] We measured the transcript levels of 36 genes expressed from the *HLA* locus nearby these signals[22–25] in patient-derived cervical samples and in a cervical epithelial cell line. We report on *HLA* genes that were associated with SNP genotypes, HPV infection status and/or in vitro inflammatory response.

2 | METHODS

2.1 | Patients

The Cervigen Study is a multi-centric hospital based cervical cancer and dysplasia series originating from nine German hospitals in Hannover, Wolfsburg, Jena,

Erlangen, Dresden, Halle, Munich, Berlin, and Bad Münder, as described previously.[18] 3764 samples were used for the present case–control analysis, after exclusion of patients with known non-European ancestry. We included 1122 cases with invasive cervical cancer and 1408 cases with cervical dysplasias (241 low-grade dysplasias and 1167 high-grade dysplasias). Additionally, 1196 healthy females from Hannover Medical School were taken as population controls. Age distribution and HPV positivity rates have been described previously.[18,26] Genomic DNA extracted from 5 mL EDTA blood via the standard phenol-chloroform method was taken for genotyping. Median age at diagnosis was 44 years (range 17–94 years) for patients with invasive cervical cancer and 31 years (range 16–79 years) for patients with cervical dysplasia, compared to a median age at recruitment of 32 years (range 18–86 years) for healthy female controls (voluntary participation at our center, with informed consent). Methanol-fixed cervical tissue smears were taken from a smaller cohort ($n = 303$) of women undergoing colposcopy at Hannover Medical School and genomic DNA was extracted from these samples via the M24 SP robot (Abbott), as reported previously.[26] RNA was isolated from these samples as detailed below in the sub-section "Transcript analysis". Twenty-three samples were excluded due to poor quality or insufficient epithelial content (low/non-detectable expression of epithelial markers *KRT8*, *KRT18* and *EPCAM* at $C_T > 32$). Out of the remaining cohort of 280 cervical tissue samples, 78 samples were HPV positive and 202 were HPV negative. Thirty-three samples were infected by HPV16, 9 samples contained HPV18, and 36 samples had other strains of hrHPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, or 68) as determined with the RealTime High Risk HPV test on the Abbott m2000 PCR system. There was a high correlation between positive HPV status and the presence of cytological lesions: 184/202 HPV-negative samples were lesion-negative (91%), while 54/78 HPV-positive samples were lesion-positive (69%).

2.2 | SNP genotyping

Fluidigm® SNPtype assays with allele-specific probes labelled with FAM® or HEX® dyes were designed for genotyping the nine variants rs17190106,[9] rs535777,[9] rs1056429 (proxy for rs1053726[9,11]), rs2763979,[9] rs3117027[7,14] and its proxy rs3130214, rs9277610 (proxy for rs3130196[7,14]), and rs143954678 and rs113937848 (proxies for rs6938453[10]), representing six independent GWAS signals. Fluidigm SNPtype assay IDs, MAF in Europeans (1000Genomes[27]), and alleles genotyped are listed in Table S1. Two non-template negative controls were taken per run. Example cluster plots are shown in Figure S1. The additional variant rs9272117 has been genotyped in genomic DNA from the cervical tissue smears previously and the genotypes were available for expression quantitative trait locus (eQTL) analysis in this study.[18]

2.3 | Statistical analysis

All the variants were tested for deviation from Hardy–Weinberg equilibrium (HWE) in Goodness-of-fit chi-square tests and variants passing HWE passed that they were taken for further statistical analysis. Call rates for the variants are given in Table S1. We performed logistic regression to calculate odds ratios (ORs), p-values and 95% confidence intervals (CIs) under an additive model, with case–control status as the outcome and variant genotype as the predictor variable, using STATA17. Odds ratios are given relative to the common homozygous genotype for all variants (note the MAFs in Table S1). Analyses were restricted to participants with questionnaire-based European ancestry. We performed an overall analysis of cervical disease versus disease free controls and performed stratified analyses for the low-grade dysplasia (CIN1 + CIN2 cases at age < 30 years (CIN2<30)), high-grade dysplasia (CIN2 cases at age ≥ 30 years (CIN2≥30) and CIN3 patients), invasive cancers, high-grade dysplasia & invasive cancers, high-grade dysplasia & invasive cancers when HPV16, HPV18 or other hrHPV positive, squamous epithelial cell carcinomas, and adenocarcinomas. In regard of multiple testing for 6 SNPs and 10 comparisons, a Bonferroni corrected p-value of $p < 0.0008$ would be considered statistically significant. However, as the candidate variants came with prior evidence from a previous GWAS, an association with $p < 0.05$ and the same direction of effect was taken as confirmatory evidence in this replication study. Stepwise conditional regression analysis was performed in STATA17 to identify independent signals at a locus (where linked SNPs were present). Haplotype analysis for SNPs close to each other was performed using Haploview.[28]

2.4 | Transcript analysis

Total RNA was extracted using Trizol reagent (peqGOLD TriFast™) from methanol-fixed cervical tissue samples of 303 healthy females who underwent routine HPV testing at Hannover Medical School.[13] 1ug RNA was reverse transcribed into cDNA using the ProtoScript® II First Strand cDNA Synthesis Kit (New England BioLabs).

Fluidigm® DeltaGene assays were designed for 36 genes at the *HLA* locus (Fluidigm Assay IDs for each gene are given in Table S2), for epithelial marker genes *KRT8*, *KRT18* and *EPCAM*, and for housekeeping genes *B2M* and *RPL13A*. The following target genes were selected based on their physical distance from the SNP of interest or eQTL evidence from GTEx[29] whole blood or HaploReg[30] v4.2 (Supplementary Table S2, Figure 1A). Genes including *HLA-B* and *HLA-C* (classical MHC Class I genes expressing cell-surface proteins involved mainly in the endogenous antigen presentation pathway), *HLA-DRB1*, *−DRB5*, *−DBR6*, *−DQA1*, *−DQA2*, *−DQB1*, *−DQB1-AS1*, *−DPA1*, *−DPB1*, and *−DPB2* (MHC Class II genes expressed on the surface of antigen presenting cells), complement factors *C4A* and *C4B*, and BAG Cochaperone 6 (*BAG6*) (MHC Class III genes, interspersed between Class I and II, with known roles in the immune response and signalling), Lymphotoxin-beta (*LTB* or *TNFC*), Leukocyte specific transcript 1 (*LST1*), Heat shock protein genes HSP associated 1-like (*HSPA1L*) and HSP associated 1B (*HSPA1B*), signalling molecule Tenascin XB (*TNXB*), chaperone Neurogenic locus notch homolog 4 (*NOTCH4*), Pre-B-cell leukaemia transcription factor 2 (*PBX2*), Mitochondrial coiled coil domain 1 (*MCCD1*), ATPase V1 subunit G2 (*ATP6V1G2*), the RNA helicase DEAD box polypeptide 39B (*DDX39B*

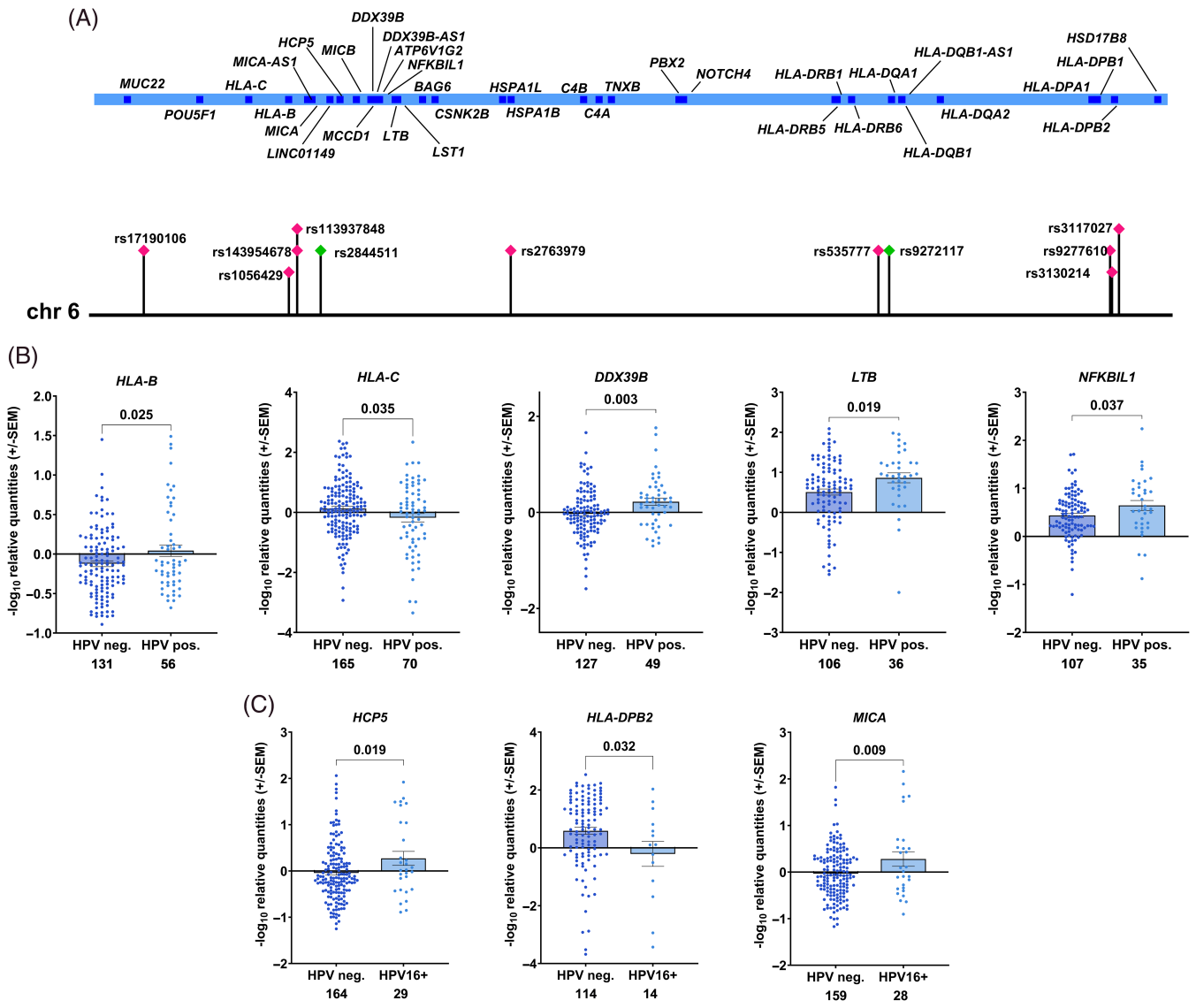

FIGURE 1 Transcript studies at the *HLA* locus in cervical tissues. (A) 36 genes and 11 SNPs at the *HLA* locus investigated in this study. SNPs marked in pink are nine novel SNPs in this study, two SNPs marked in green have been investigated in the Cervigen cohort previously. *MUC22* starts at chr6:30978251 (GRCh37) and *HSD17B8* starts at chr6:33172414 (GRCh37). Transcript expression in (B) HPV negative versus positive tissues and (C) HPV negative versus HPV16+ tissues. −log10 relative quantities (+/− Standard error of the mean (SEM)) are shown on the y-axis. Sample numbers per group are below the respective bars on the x-axis.

or *BAT1*) and its anti-sense (*DDX39B-AS1*), NF-kappa-B inhibitor-like protein 1 (*NFKBIL1*), hypervariable Mucin 22 (*MUC22*), Casein kinase II B (*CSNK2B*), and POU Class 5 Homeobox 1 (*POU5F1*) (belonging to the Class IV cytokine and interferon family of genes, regulating inflammatory response), non-coding RNAs HLA Complex P5 (*HCP5*) and *LINC01149*, the MIC (MHC class I chain related) genes *MICA*, *MICB*, *MICA-AS1* and the enzyme encoding gene Hydroxysteroid 17-beta dehydrogenase (*HSD17B8 or HKE6 or RING2*) were investigated[22–25] (Table S2, Figure 1A). The cDNA was used for RT-qPCR analysis in 48x48 integrated fluidic circuit (IFC) plates on a BioMark HD real-time PCR instrument (Fluidigm) as per manufacturer's instructions. The relative gene quantities were calculated with *B2M* and *RPL13A* as housekeeping controls using qBASE+ (Biogazelle).[31,32] Samples with poor quality or low/non-detectable expression of *KRT8*, *KRT18* and *EPCAM* at $C_T > 32$ were excluded.

Outliers were excluded via the ROUT method (1% false discovery rate) on GraphPad Prism v9.3.1. A p-value <0.05 was considered significant in the student's t-test (comparing two groups) and ANOVA (comparing three or more groups). Association of HPV status with gene expression was tested and stratified analysis was conducted for high-risk HPV subtype 16. Pearson correlation coefficients (R) were calculated for pairwise combinations of relative gene quantities using GraphPad Prism v10.

Principal component analysis (PCA) was performed on the gene expression levels to extract the first two Principal Components (PCs) for the entire cervical tissue dataset using GraphPad Prism v10, to check if any genes clustered together. These two PCs per gene were then plotted in an XY graph to visualise the genes in proximity to each other.

In order to investigate the role of variants as eQTLs, relative levels of 36 gene transcripts were tested for association with genotypes in the corresponding genomic DNA samples and examined overall as well as after stratifying into HPV positive and negative samples. In eQTL analysis, for correction for multiple testing, the Bonferroni correction method was applied by adjusting the p-value threshold by the number of tests performed. Due to the multiple testing penalty for checking a combination of 36 genes and 5 SNPs (rs9272117, rs143954678, rs17190106, rs535777, and rs1056429), only p-values below 0.00027 were considered to be *bona fide* eQTLs.

2.5 | Cell culture

Human cervical epithelial cells (HCerEpiC) from ScienCell were immortalised with the SV40 large-T antigen to generate the HCEC-T cell line. Cells were cultured in poly-L-lysine coated flasks and plates, using cervical epithelial cell medium (ScienCell) in an incubator at 37°C with 5% CO_2. Cells were seeded on a 12 well plate and 24 h after seeding, 2, 10, and 50 ng/mL of y-IFN protein (PeproTech, Gibco) was added to the medium in the respective wells, along with water added to two wells as control. Cells were harvested 24, 48, and 72 h after treatment and total RNA was isolated using Trizol reagent (peqGOLD TriFast™), followed by cDNA synthesis and gene expression analysis as above. Experiments were performed in biological triplicate with qRT-PCR experiments in technical duplicates. Statistical analysis was performed using ANOVA and a linear-by-linear trend test to check for trends across time points and varying γ-IFN concentrations in GraphPad PRISM v10.

3 | RESULTS

After wet-lab genotyping in the Cervigen cohort, variant rs17190106 showed evidence of association with overall cervical disease (OR = 0.82, 95% CI = 0.68–0.98, $p = 0.03$), low-grade dysplasia (OR = 0.64, 95% CI = 0.42–0.97, $p = 0.04$), and invasive cancer (OR = 0.69, 95% CI = 0.55–0.86, $p = 0.001$). The association was strongest for invasive squamous epithelial cell carcinoma (OR = 0.64, 95% CI = 0.48–0.85, $p = 0.002$) as detailed in Table 1.

Linked variants rs143954678 and rs113937848 ($R^2 = 1$) both showed evidence of association with overall cervical disease (OR = 0.86, 95% CI = 0.76–0.98, $p = 0.02$, for rs143954678; and OR = 0.87, 95% CI = 0.78–0.98, $p = 0.03$, for rs113937848) as well as with invasive cancer and squamous carcinomas. Variant rs1056429 showed evidence of association with overall cervical disease (OR = 0.84, 95% CI = 0.73–0.97, $p = 0.02$), high-grade dysplasia, invasive cancer and squamous carcinoma. Variant rs535777 did not associate with overall disease but showed evidence of association with low-grade dysplasia and adenocarcinomas (OR = 1.49, 95% CI = 1.1–2.01, $p = 0.01$, for LSIL; OR = 1.63, 95% CI = 1.17–2.27, $p = 0.004$ for adenocarcinoma) (Table 1). The variants rs3117027 and rs9277610 did not associate with overall disease but showed evidence for association with high-grade dysplasia and invasive disease when stratified for HPV16 (Table S3). The variants rs2763979 and rs3130214 did not show any associations at $p < 0.05$ in our cohort (Table S3).

In stepwise conditional regression analysis for the three weakly linked variants rs9277610, rs3130214 and rs3117027 ($R^2 = 0.1$–0.2), none of them became an independent significant predictor of cervical cancer risk (Table S4A) and no particular haplotype was found to

TABLE 1 Results after logistic regression analyses from the genetic case control study. Cervical intraepithelial neoplasia was grouped into LSIL/low-grade(CIN1 + CIN2 < 30 years) and HSIL/high-grade (CIN2 ≥ 30 years + CIN3) subgroups. Invasive cervical cancer was further separated into squamous epithelial cell carcinoma or adenocarcinoma. High-risk dysplasia (CIN2 ≥ 30 years + CIN3) and invasive cancer were also combined together. HSIL & invasive were further stratified by HPV status (hrHPV 16, 18 or other hrHPV). CI, 95% confidence interval; OR, odds ratio for minor allele; *p*, *p* value from logistic regression analysis.

	rs17190106				**rs143954678**				**rs113937848**				**rs1056429**				**rs535777**			
Stratum	**nCases**	**nControls**	**OR (95% CI)**	***p***	**nCases**	**nControls**	**OR (95% CI)**	***p***	**nCases**	**nControls**	**OR (95% CI)**	***p***	**nCases**	**nControls**	**OR (95% CI)**	***p***	**nCases**	**nControls**	**OR (95% CI)**	***p***
Overall	2517	1196	0.82 (0.68–0.98)	**0.03**	2570	1016	0.86 (0.76–0.98)	**0.021**	2571	1193	0.87 (0.78–0.98)	**0.03**	2570	1194	0.84 (0.73–0.97)	**0.016**	2491	1011	1.06 (0.89–1.26)	0.49
LSIL (CIN1 + CIN2 < 30y)	235	1196	0.64 (0.42–0.97)	**0.036**	241	1016	0.83 (0.64–1.06)	0.136	241	1193	0.84 (0.65–1.07)	0.161	241	1194	0.94 (0.71–1.24)	0.657	230	1011	1.49 (1.1–2.01)	**0.01**
HSIL (CIN2 ≥ 30y + CIN3)	1110	1196	0.98 (0.79–1.21)	0.84	1167	1016	0.89 (0.77–1.03)	0.115	1167	1193	0.90 (0.79–1.04)	0.147	1167	1194	0.85 (0.72–1.00)	**0.048**	1095	1011	0.97 (0.79–1.19)	0.77
Invasive	1097	1196	0.69 (0.55–0.86)	**0.001**	1121	1016	0.85 (0.74–0.99)	**0.034**	1122	1193	0.86 (0.75–0.99)	**0.04**	1121	1194	0.82 (0.70–0.97)	**0.023**	1091	1011	1.07 (0.87–1.30)	0.52
HSIL + invasive	2207	1196	0.83 (0.69–0.99)	**0.049**	2288	1016	0.87 (0.77–0.99)	**0.034**	2289	1193	0.88 (0.78–1.00)	**0.04**	2288	1194	0.83 (0.72–0.96)	**0.012**	2186	1011	1.02 (0.86–1.22)	0.84
HSIL + invasive HPV16 + ve	294	1196	0.95 (0.68–1.32)	0.75	326	1016	0.70 (0.56–0.89)	**0.003**	326	1193	0.72 (0.58–0.91)	**0.005**	327	1194	0.90 (0.71–1.15)	0.415	290	1011	1.08 (0.79–1.46)	0.62
HSIL + invasive HPV18 + ve	98	1196	0.78 (0.43–1.38)	0.39	113	1016	0.75 (0.53–1.08)	0.120	113	1193	0.76 (0.53–1.09)	0.137	113	1194	0.88 (0.59–1.30)	0.514	96	1011	1.74 (1.15–2.64)	**0.009**
HSIL + invasive HPVOther+ve	73	1196	0.98 (0.53–1.79)	0.94	82	1016	0.69 (0.45–1.06)	0.090	83	1193	0.69 (0.45–1.06)	0.087	82	1194	0.89 (0.56–1.40)	0.609	72	1011	1.38 (0.83–2.30)	0.22
Adenocarcinoma	182	1196	0.84 (0.54–1.30)	0.44	197	1016	1.13 (0.88–1.45)	0.349	198	1193	1.13 (0.89–1.45)	0.317	197	1194	0.93 (0.68–1.26)	0.627	181	1011	1.63 (1.17–2.27)	**0.004**
Squamous carcinoma	689	1196	0.64 (0.48–0.85)	**0.002**	733	1016	0.77 (0.65–0.91)	**0.002**	733	1193	0.78 (0.66–0.92)	**0.003**	732	1194	0.78 (0.64–0.94)	**0.011**	689	1011	0.96 (0.76–1.22)	0.74

provide an increased risk at $p < 0.05$ (Table S5A). Since this signal did not replicate, we did not follow it in further eQTL analysis. In conditional regression and haplotype analysis, we also tested the linked variants rs143954678 and rs113937848 representing rs6938453,[10] and sparsely linked with the well-known *MICA* variants rs2516448/rs2844511[7,14,18] ($R^2 \sim 0.2$). We found that rs143954678 and rs113937848 correlated highly with each other, and the slightly stronger risk variant rs143954678 was taken as representative for detailed eQTL analysis (Table S4B). Haplotype analysis revealed evidence of association with overall disease for a combination of the rare alleles of rs143954678 and rs113937848 with the common allele of rs2844511 ($p = 0.01$, Table S5B).

In order to find out whether the variants associated with gene transcript levels in cervical tissues, we transferred the replicating novel variants from above (rs17190106, rs143954678, rs535777, and rs1056429) to further eQTL analyses and also included a known variant rs9272117 that was associated with overall cervical disease and high-grade dysplasia in this cohort as reported previously (Figure 1A).[18] We measured the levels of 36 *HLA* gene transcripts adjacent to these loci at chr6p21 in 280 cervical tissue samples with confirmed epithelial content and known HPV status (Figure 1A). The transcript levels for five genes showed evidence of association with HPV status: The levels of *HLA-C* were decreased in HPV positive samples ($p = 0.04$) whereas *HLA-B*, *DDX39B*, *NFKBIL1*, and *LTB* levels were higher in HPV positive samples ($p = 0.03$, 0.002, 0.04, 0.02, respectively), though these marginal *p*-values do not withstand multiple testing correction (Figure 1B). In stratified analysis based on HPV subtype, the levels of *MICA* and *HCP5* were found to be higher in HPV16+ samples ($p = 0.009$, 0.02, respectively), whereas *HLA-DPB2* was found to be lower in HPV16+ samples ($p = 0.03$) (Figure 1C).

Transcript correlation analysis revealed strong correlation (Pearson's R) for the transcript levels of several genes (Figure 2A, Table S6), with neighbouring genes also clustering together in PCA (such as *C4A* and *C4B*, *HLA-B* and *HLA-C*, *HLA-DBP1* and *HLA-DBP2*) (Figure 2B). There were marked changes in the correlation pattern in HPV positive as compared to HPV negative samples, indicating that HPV infection may induce transcriptional dysregulation (Figure 2D,C; Table S6).

In eQTL analysis, multiple SNP-gene pairs were tested overall, and in HPV positive and negative tissues. We tested the eQTL effects for a combination of 36 genes and 5 SNPs, and found suggestive eQTL evidence for several variants at $p < 0.05$ (Supplementary Table S7). When only *p*-values below 0.00027 were considered to pass the multiple testing threshold, the variant rs9272117 (proxy for the susceptibility variant from a Swedish cervical cancer GWAS, rs9272143[7]) remained significant as an eQTL for *HLA-DRB6* ($p = 1.9$X10E-5 overall and $p = 7.4$x10E-5 in HPV negative tissues), while rs1056429 remained an eQTL for *HLA-DRB5* ($p = 2.5$x10E-4) overall, and rs535777 for *HLA-DRB1* ($p = 2.7$x10E-4) in HPV negative tissues. (Figure 3A–C). These strong eQTLs found in the cervical epithelial cells differed from the predicted eQTLs in GTEx[29] and DICE[33] databases in other cell types (Table S8 and Table S9).

We tested whether some eQTL variants may also modify the observed correlation pattern for transcripts in the *HLA* region. The genotype of rs9272117 that was associated with *HLA-DRB6*, and less significantly with *HLA-DRB5*, *MCCD1*, *MUC22*, and *PBX2* transcript levels (Table S7), was found to impact multiple gene correlations overall, and in HPV negative or positive samples (Figure 3D). For example, the correlation between the levels of *HLA-DRB5* and *HLA-DRB6* was markedly decreased when the rare allele of rs9272117 was present, with a similar effect seen in HPV positive samples as compared to HPV negative samples (Figure 3D, Table S10).

In a complementary approach, we studied the expression of the same set of HLA gene transcripts in a patient-derived cervical epithelial cell line (HCEC-T, free of HPV infection and dysplastic changes) under basal and inflammatory conditions. In order to elucidate whether some of the genes that were differentially regulated by HPV status or genomic risk variants in our patient cohort may also represent cytokine-responsive genes, we performed transcript analyses for the 36 *HLA* genes after exposure to a proinflammatory cytokine, γ-interferon. In HCEC-T cells treated with increasing doses of γ-IFN, we observed a dose-dependent up-regulation of *HCP5*, *HLA-B*, *HLA-C*, *HLA-DQB1*, *HLA-DRB1*, and *HLA-DRB6* levels ($p < 0.05$, Figure 4), with the strongest effects seen at 48 hours after addition of the reagent compared to untreated cells. *HSPA1L* levels showed a γ-IFN dose-dependent decrease in these cells (Figure 4). There was some overlap between genes that appeared to be regulated through genomic variants (including borderline eQTLs) or HPV status and through γ-IFN treatment (dose-specific at any time point) (Figures S2–S6). For example, *HLA-DRB1* levels that were upregulated in carriers of the rare allele of rs535777 selectively in HPV positive tissues (Figure 3), were also induced in γ-IFN treated HCEC-T cells in a dose-dependent manner after 48 and 72 h of treatment (Figure 4).

In summary, we have replicated four additional signals at the *HLA* locus in our case–control series, indicating that these might represent true, independent genetic risk factors. We found novel cervical epithelium-specific eQTLs and strong gene correlations at these loci in a patient-derived cDNA cohort, and identified *HLA* genes whose expression was associated with HPV status and/or induced upon γ-IFN treatment in a cervical epithelial cell

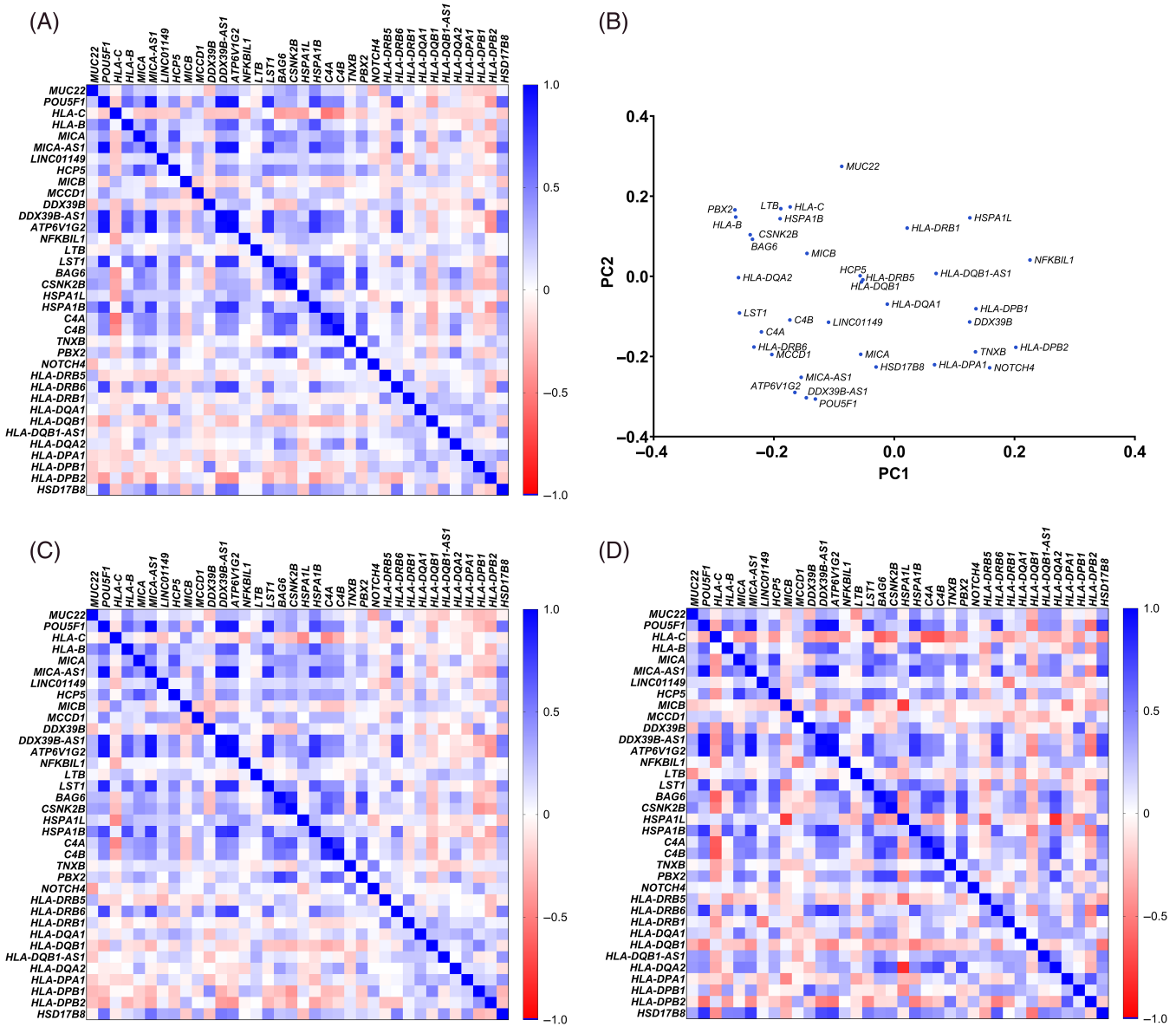

FIGURE 2 Transcript correlation at the *HLA* locus. Pearson correlation R values are plotted between genes (A) Overall, and restricted to (c) HPV negative tissues or (D) HPV positive tissues. Negative correlation ($R = -1$) is shown in deep red colour, whereas positive correlation (R = 1) is shown in deep blue colour. (B) Gene clusters seen in all tissues after principal component analysis. PC1 and PC2 make up the two axes.

line, as well as genes that were not expressed in the latter model. Taken together, these findings advance our knowledge of an interplay between genomic risk factors, HPV infection and inflammation-based activation of genes at the 6p21.32–33 cervical cancer susceptibility locus.

4 | DISCUSSION

Multiple variants at the *HLA* locus have been identified in CC GWASs, and a handful have been replicated in independent populations. The first aim of our study was to investigate whether the *HLA* variants independently associate with cervical cancer and dysplasia risk in the German Cervigen case–control series and to identify genes regulated through the confirmed genomic risk loci. A second aim was to investigate which of the genes located at the confirmed loci were responsive to inflammatory stimulation in cervical epithelial cells and whether there was overlap between those and HPV- or SNP-associated genes in cervical tissue. This complementary approach may distinguish inflammatory regulation from direct regulation through HPV proteins or dysplastic changes, and thus should help interpret the results that we obtained from HPV−/+ patient tissues.

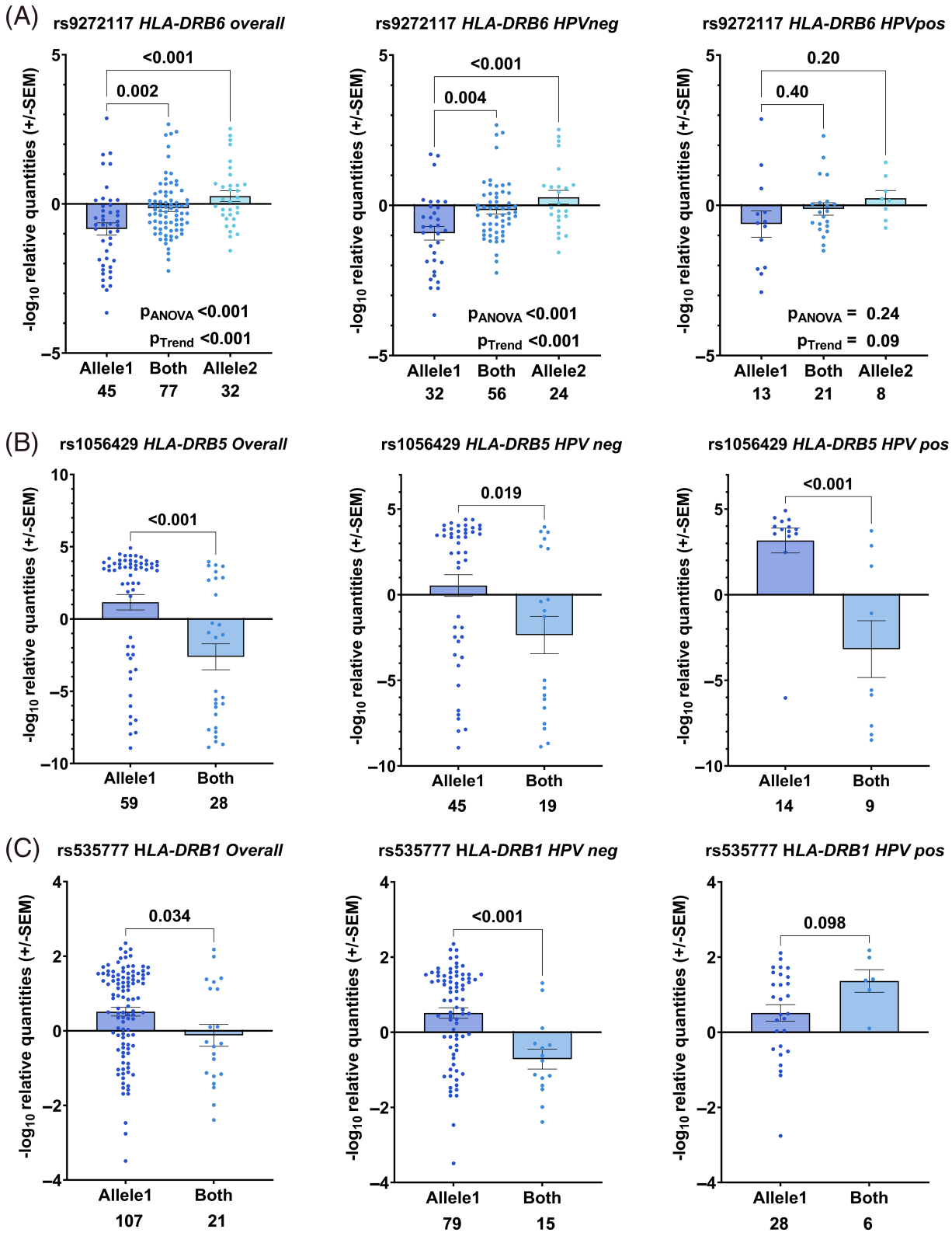

FIGURE 3 eQTL analysis in cervical tissues. −log10 relative quantities (+/−SEM) are shown on the y-axis, together with SNP genotype on the x axis for (A) rs9272117, (B) rs1056429, and (C) rs535777. Panels from left to right are all samples, HPV negative and HPV positive samples. Sample numbers per group are below the respective bars on the x-axis. T-test was performed between two groups or ANOVA followed by a linear trend test between three groups, with the common genotype as the control. (D) Pearson correlation R values are plotted between genes that showed evidence to be eQTLs with rs9272117 (A) Overall, and restricted to (c) HPV negative tissues or (D) HPV positive tissues. Panels from left to right indicate the genotype of rs9272117: CC, CT or TT. Negative correlation ($R = -1$) is shown in deep red colour, whereas positive correlation ($R = 1$) is shown in deep blue colour. Missing values are indicated with a black X through the white box.

(D)

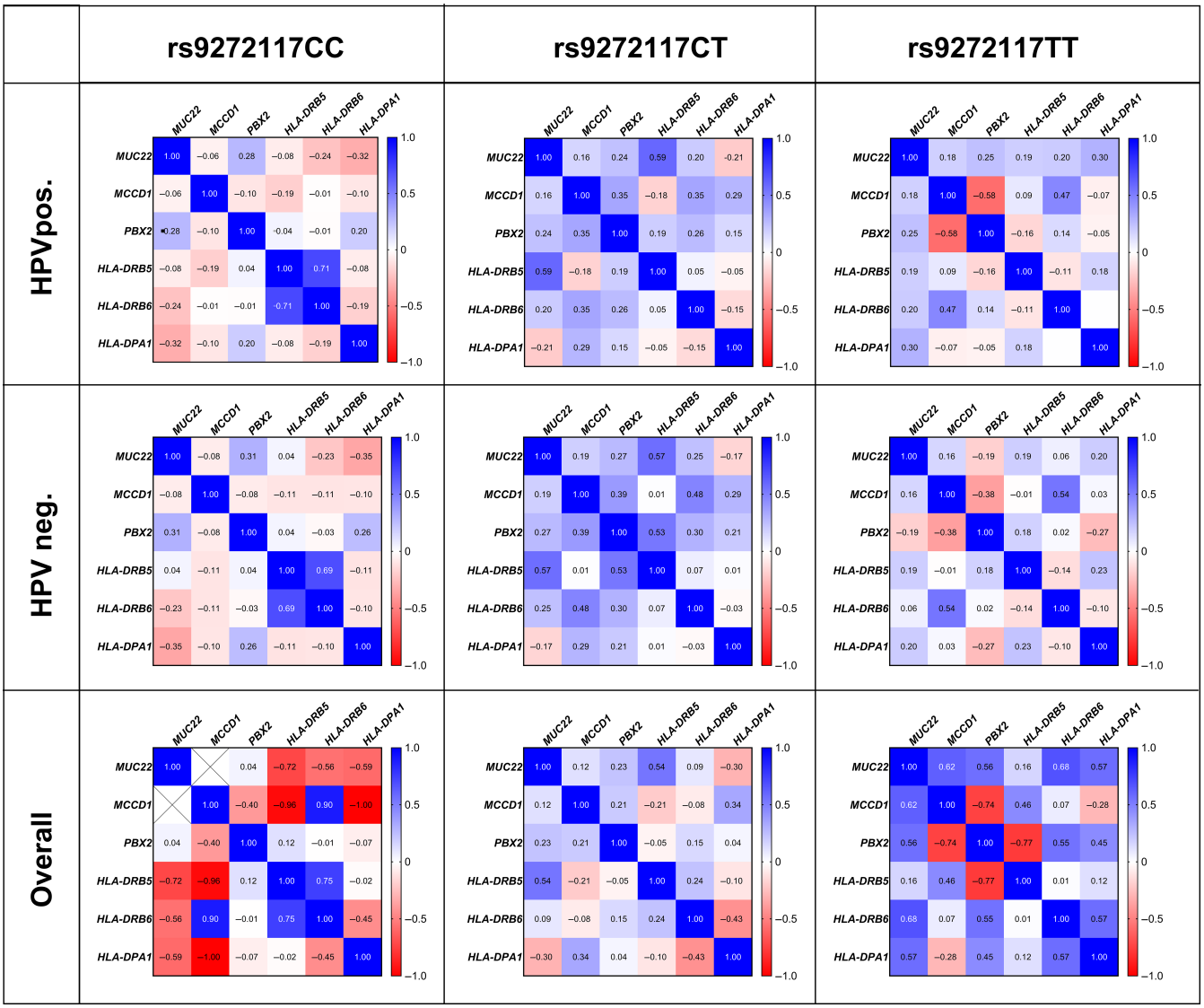

FIGURE 3 (Continued)

Among the nine lead variants from six GWAS signals newly tested, rs17190106 (G > A) close to *MUC22* and *HCG22*[9] replicated in our cohort at $p < 0.05$ and associated with overall cervical disease as well as squamous carcinomas. Although this association would not withstand correction for multiple testing, it can be taken as confirmatory evidence given the prior GWAS association in a different population. In a previous report, the allele G had been identified to increase the risk for cervical, lung, and rectal cancer whereas it was protective for cancer of the bladder, oral cavity, and pancreas, among others.[9] We replicated the findings from this initial study as the minor allele ("A") was found to be protective in our cohort. This SNP was predicted to be an eQTL for a novel lncRNA transcript *XXbac-BPG181B23.7* (*lnc-HLA-B-2:3*) in GTEx[29,34] in whole blood. In our cervical tissue series, we found mild eQTL evidence for complement genes *C4A* and *C4B*, with the rare allele decreasing the levels of the genes. Deficiency or decrease in these gene products is known to foster autoimmunity[35] and predict an increased survival in renal carcinoma.[36] Increased levels of C4b have been shown in pancreatic cancer compared to normal tissues.[37] Complement proteins C4A and C4B bind selectively more to amino group containing or hydroxyl group containing antigens, respectively.[38] While complement proteins are necessary in the immune response against pathogens, cancer cells may utilise and activate the complement pathway to promote tumour proliferation and metastasis.[39] Whether the protective rare allele of this SNP may impact survival in cervical cancer, is as yet unknown.

The signal underlying rs1056429 (genotyped as a proxy for rs1053726[11] and linked to rs9266766[9] at R^2 >0.3), close to *HLA-B*, was initially identified in a pan-

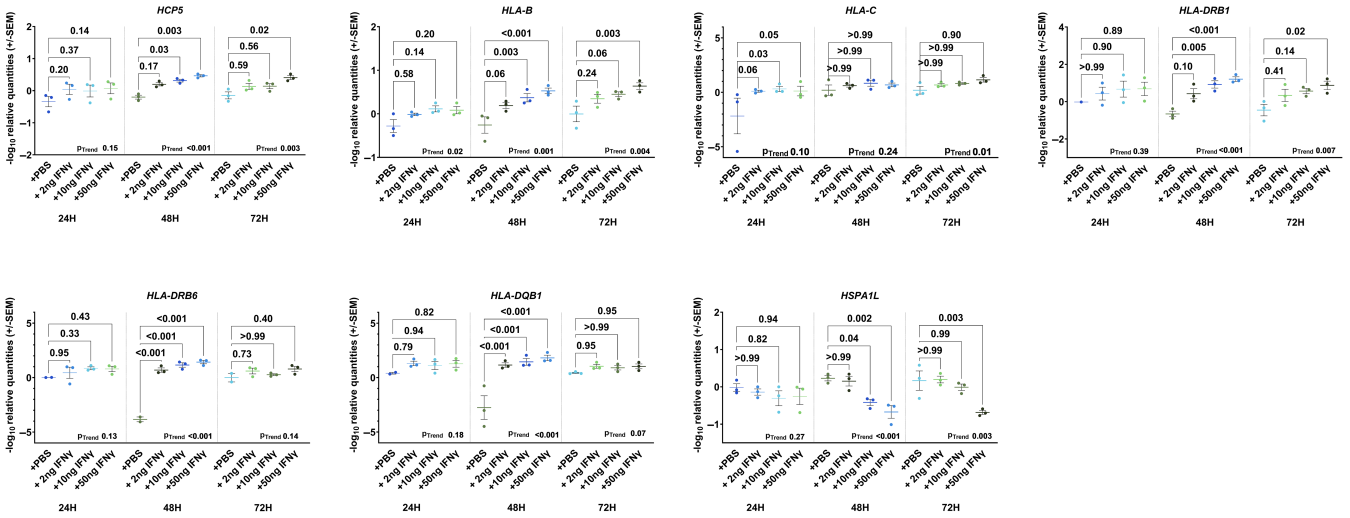

FIGURE 4 Transcript expression in cervical epithelial cell line HCEC-T after treatment with 2, 10, and 50 ng of γ-IFN for 24, 48 and 72 h. Genes shown *HCP5*, *HLA-B*, *HLA-C*, *HLA-DRB6*, *HLA-DQB1*, *HLA-DRB1*, and *HSPA1L*. −log10 relative quantities (+/−SEM) are shown on the y-axis with treatment and time on the x-axis. Each experiment was performed in biological triplicate with technical duplicate for each qRT-PCR. *p* values shown after ANOVA followed by a linear trend test between three groups, with the untreated cells of that time point as respective control.

cancer GWAS analysis[9] and the common allele "G" was found to increase the risk for cervical cancer in that study. The rare allele "G" of the linked variant rs1053726 from a cervical dysplasia GWAS meta-analysis,[11] showed a correspondingly decreased risk. For the linked variant rs1056429 (G > A, $R^2 = 0.53$), in our study, the rare allele "A" showed a similar decreased risk for dysplasia and cervical cancer and associated with decreased levels of *HLA-DRB5* in cervical tissues. Previous studies have identified *HLA-DRB5* alleles that associated with reduced squamous cervical carcinoma risk although it was suggested that the underlying susceptibility at this locus may arise from linkage with the *HLA-DRB1* locus.[19] However, our eQTL analyses point to a regulation of *HLA-DRB5* levels in epithelial cells. HLA-DRB5 protein expression is documented to be high in cervical malignant tissues in the human protein atlas (https://www.proteinatlas.org/).[40] HLA-DRB5, belonging to the class II MHC molecules, is primary involved in antigen presentation. A study reported a possible epistatic role of DRB5 modifying the T-cell response induced by DRB1 alleles in multiple sclerosis.[41]

Multiple *HLA-DRB1* alleles have been investigated in cervical cancer risk so far, with reported cis-eQTLs implicated in conveying susceptibility.[17,42–46] rs35777 (G > A), near *HLA-DRB1*, identified in a pan-cancer analysis[9] was associated with an increased risk of cervical cancer. In our case–control series, the rare allele associated with low-grade dysplasia but also adenocarcinomas. We also report on decreased levels of *HLA-DRB1* in HPV negative tissues from rare allele carriers but, interestingly, this eQTL effect reversed in HPV positive tissues. An upregulation of *HLA-DRB1* after HPV infection is also supported by *HLA-DRB1* upregulation seen in our cervical epithelial cell line upon γ-IFN treatment. This result is different from previous results in HeLa cells where γ-IFN induced HLA transcripts were limited to HLA class I transcripts[47] or HLA-DRA.[48] However, HeLa might not be fully representative of the premalignant state after HPV infection, and our non-malignant cellular model suggests that cervical epithelial cells may enhance the expression of further HLA class II molecules in response to infection which may give rise to an improved adaptive response to the infectious agent and increased viral clearance.[49,50] It remains to be tested if genetic risk variants act synergistically with the risk of an HPV infection, or at different stages of the disease and contribute separately towards advancing invasive cancer. By comparison with the immune escape phenotype of MHC class I deficient neoplasms,[51,52] the epithelial impact of MHC class II including HLA-DR molecules has been less well defined. However, HLA-DR expression in epithelial cancer cells has been correlated with T cell infiltration,[53] indicating that HLA-DR molecules play an important role in presenting exogenous antigens from tumour cells to CD4+ T lymphocytes, the T-helper cells. Consistent with this, tumour specific HLA-DR expression is associated with favourable outcomes in cancer patients.[54] While the alpha chain, DRA, is common to all heterodimers, different beta chains constituted of DRB1, DRB3, DRB4 or DRB5 contribute to MHC class II heterogeneity. Decreased HLA-DRB1 expression has been reported during colorectal cancer development in the epithelium and

stromal cells, and was associated with decreased survival.[55] Additionally, HLA-DRB1 has been reported to be fucosylated and overexpressed on the surface of melanoma cells in an antitumor therapy mechanism that results in cancer suppression via an increase of intratumoral T-cells.[56] Our results suggest that HLA-DRB1, which was induced in HPV-positive dysplasia samples and in interferon-stimulated epithelial cells, may serve a similar immune defence mechanism in cervical tissue.

rs143954678 (C > delC) and rs113937848 (T > A) (taken as proxies for rs6938453,[10] R^2 ~ 0.8), represented a secondary signal[10] identified at the *MICA* locus after rs2516448/rs2844511.[7,14,18] These two variants are only very weakly correlated with the main signal rs2516448/rs2844511 (R^2 ~ 0.2), however, stepwise conditional regression analysis indicated that they did not independently contribute to the risk in our study. The "A" allele of rs6938453 (T > A) has been associated with a decreased risk for CIN3 and invasive cancer in a UK biobank based cohort.[10] For the two alternative variants that we tested in our cohort, the rare alleles similarly showed a protective effect. Notably, rs143954678 also shows some evidence to be associated with high-grade dysplasia ($p = 2.8$x10E-03) and malignant cervical cancer ($p = 6.0$x10E-3) in the Finnish biobank cervical cancer GWAS (https://r8.finngen.fi/variant/6:31372159-TC-T). Although no eQTLs could be determined above the multiple-testing threshold, in HPV positive tissues, rs143954678 weakly associated with *MICB* and *HLA-DPA1* levels. It was also predicted to be a strong eQTL for lncRNA transcript *XXbac-BPG181B23.7* (*lnc-HLA-B-2*:*3*) in GTEx in whole blood, but we did not test this non-coding RNA in the current study. Previously, rs2516448 was linked to a frameshift mutation A5.1 in the *MICA* gene and rs2844511 was found to decrease *MICA* levels in HPV positive tissues.[18,45] Our haplotype analyses showed an association with disease risk only for the rare alleles of rs143954678 and rs113937848 combined with the common allele of rs2844511, indicating that both signals may be needed for a full effect or that the risk haplotype tags another rare variant.

We also performed extended eQTL analysis for the first identified CC GWAS signal at the *HLA* region,[7] represented by rs9272117 (as proxy SNP for rs9272143 with $R^2 = 0.9$). The variant rs9272117 stood out in apparently regulating multiple genes and its genotype also impacted the correlation between the transcript levels of these genes, suggesting that it may affect a regulatory element with long-range effects across the *HLA* region. In particular, the protective rare allele of rs9272117 strongly associated with increased *HLA-DRB6* levels and decreased levels of *HLA-DRB5*, regardless of HPV status. A dose-dependent increase of *HLA-DRB6* levels was also seen in the HCEC-T cells upon addition of γ-interferon suggesting that this gene is upregulated under inflammatory conditions in response to a viral infection. This was unexpected since *HLA-DRB6* is regarded as a pseudogene,[22,57,58] suggested to evolve from early DRB-precursor genes upon the insertion of an unknown Alu or transposable element into intron 1 and deletion of exon 1, rendering it a pseudogene without promoter available.[59] There is a lack of clarity on the pseudogenic nature of *HLA-DRB6* as initial suggestions were challenged by studies that detected low levels of prematurely spliced and even mature *HLA-DRB6* mRNA in a human B-cell line.[60,61] The latter findings were independently confirmed[62] and it was suggested that the retroviral element inactivating the original promoter of *DRB6* contained a new cryptic promoter and a new exon 1 enabling *HLA-DRB6* expression.[62] In our analysis, we detected *HLA-DRB6* at the cDNA level in our cervical epithelial tissue specimens and find that cervical cancer risk variant rs9272117 may affect the expression of this gene transcript. It remains to be tested whether this translates into membrane expression of the protein or into truncated, soluble versions of DRB6 (similar to MICA or HLA-G). However, evidence has been presented that DRB6 protein can be detected as a transmembrane protein and may be involved in immune signalling or homeostasis.[63] Being closely associated with the functional *DRB4* gene,[64] it is likely that *DRB6* may perform similar functions due to sequence homology and structural similarities.[65] Alternatively, non-coding genes have been suggested to be involved in transcriptional regulation as lncRNA, miRNA, or mRNA sponges/decoys,[66] however any RNA-interaction studies for *HLA-DRB6* remain to be performed. By comparison, *HLA-DRB5* basal levels were too low in HCEC-T cells to be reliably assessed for further suppression.

HCEC-T served as a newly generated cell culture model of cervical epithelial cells to test which HLA molecules are induced in response to γ-IFN. Our results are in line with previous work[67] since it is known that certain *HLA* genes get activated upon γ-IFN addition due to the presence of responsive elements in the promoters.[68,69] A study reported that HLA upregulation happens in immune cells in the cervix as opposed to the keratinocytes,[70] however it is now suggested that the response occurs in both cell types in the cervix.[71] The upregulated molecules may then activate mediators in the adaptive immune response.[67,72–74] HPV is reported to have defensive mechanisms in place to decrease HLA expression in keratinocytes such as by methylation or integration or reduction of γ-IFN mediated upregulation of HLA expression.[75–80] The loss of MHC Class I expression and resulting "immune desertification" has been noted as a common feature of therapy-resistant tumours.[81] The development of invasive metastatic cervical cancer has

also been linked to allelic loss of HLA Class I[82] or reduced HLA Class I and II expression and thereby decreased cytotoxic death of cancer cells.[83,84] The down-regulation of certain HLA molecules due to prolonged HPV infection may enable the development of invasive cervical disease.

Our study has certain limitations as we note here: we tested the index variants at the listed novel GWAS loci, which may not necessarily be the true causal variants, and our genetic study was limited to participants of European ancestry. Fine-mapping studies would be needed to determine the true causal variant as eQTL evidence in the tissue of interest might point to one regulatory variant among further linked ones.[85] This further invites validation studies in larger cohorts. Our cell culture work was limited to the HCEC-T cell line that was established for this purpose, and one inflammatory cytokine. This might partially but not fully mimic the inflammatory environment after HPV infection. Our panel of 36 genes tested for eQTLs included the most prominent *HLA* genes but several non-coding and pseudo-genes from that genomic region have remained untested. Furthermore, due to the high correlation between HPV and lesion status in our cervical tissue series, we could not clearly distinguish between HPV-induced regulatory events and those that may be secondary to dysplastic changes. It also remains to be determined whether the eQTL effect mediated by the risk variants plays a role at an advanced stage of the disease, when the persistent infection has activated multiple immune evasion mechanisms in place.

In the current study, we provide evidence that *HCP5*, *HLA-B*, *HLA-C*, *HLA-DQB1*, *HLA-DRB1*, and *HLA-DRB6* are up-regulated in a model of cervical epithelial cells by inflammatory cytokine treatment, mimicking an antiviral response. This extends the list of relevant targets to both the HLA Class I and HLA Class II regions and identifies cervical epithelial cells as mediators of the immune response. Future studies can determine whether targeted therapy methods tailored to enhancing HLA-mediated anti-tumour response[81,86–88] can be used as a veritable treatment option for cervical cancer.

5 | CONCLUSIONS

In brief, we validated four further GWAS signals at the *HLA* locus in our case control study and provided evidence for two of these new signals to be eQTLs in our cervical epithelial cohort. We established a cell line for investigating genes that respond to cytokines and may serve as a model to study cervical infection and cancer development. Combined analyses of risk variants after association and eQTL studies with the HPV- and/or IFNγ-mediated effects indicated a regulatory role of some GWAS variants and suggests the involvement of both HLA I and HLA II region gene expression in cervical cancer risk.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest. Peter A. Fasching conducts research funded by Amgen, Novartis and Pfizer and received Honoraria from Roche, Novartis and Pfizer. None of these sponsors had any role in the design, data acquisition, analysis or interpretation of results in this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The Ethics committee of Hannover Medical School approved this study (Votes No. 441 and 10737). The samples and corresponding clinical data were obtained after informed consent in accordance with German medical council regulations.

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