1. Introduction

Persistent infection with an oncogenic human papillomavirus (HPV) type is an essential requirement for the development of cervical cancer [1]. However, most HPV infections are cleared by the immune system within one to two years after initial infection [2]. Of the currently known oncogenic HPV types, HPV16 and HPV18 cause around 70% of all cervical cancer cases worldwide [3].

The progression from initial HPV infection to cervical cancer occurs via precursor lesions (cervical intraepithelial neoplasia, CIN grade 1 to 3) and may take decades [4,5]. Treatment of CIN2/3 lesions is performed by ablative or excisional treatment. Women treated for CIN2/3 lesions have a 5–15% risk of developing recurrent high-grade lesions (rCIN2/3) within two years post-treatment [6,7]. Consequently, women undergoing treatment for CIN2/3 are closely monitored in the post-treatment period, before they return to a regular screening routine [7,8]. In the Netherlands women are tested by cytology and HPV co-testing at 6, 12 and 24 months post-treatment [9]. The 12 months visit can be omitted when the 6-month visit shows absence of HPV and normal cytology. After three consecutive negative co-tests, the woman is referred back to screening programme [7,9].

rCIN2/3 represents a heterogeneous group of lesions, consisting of either residual CIN2/3 or a new CIN2/3 lesion. Residual CIN2/3 is a possible consequence of incomplete excision of the original CIN2/3 lesion, characterized by the same HPV type in the rCIN2/3 as in the baseline lesion. A new CIN2/3 lesion would occur from a newly acquired HPV infection by a different type (type switch), or an infection with a different variant of the same HPV type present in the baseline lesion [10]. Post-treatment surveillance should ideally differentiate residual from incident lesions, as women with residual lesions are in need of immediate treatment. Women with incident lesions may benefit from a more conservative approach due to a lower cancer risk [11].

In a recent multicenter post-treatment surveillance study, most baseline CIN2/3, rCIN3 and a subset of rCIN2 harbored HPV16 by genotyping [11]. From a clinical perspective, this poses the question whether the rCIN2/3 was caused by a newly acquired HPV16 infection, or by the same infection detected at baseline, which resulted in CIN2/3. Here, we employ whole genome next-generation sequencing of HPV16 to identify and compare consensus variants in paired baseline CIN2/3 and rCIN2/3 cervical scrapes.
Table 1: Characteristics of patients included in this study.

| Study number | Histology at 6 or 12 months post treatment | Months post-disease diagnosis | Nucleotide changes |
|--------------|-------------------------------------------|-------------------------------|-------------------|
| 10           | CIN2                                      | 7                            | 3723T > G         |
| 203          | CIN2                                      | 5                            | –                 |
| 402          | CIN3                                      | 14                           | –                 |
| 410          | CIN3                                      | 11                           | –                 |
| 422          | CIN3                                      | 12                           | 3800C > G, 7702G > C |
| 638          | CIN3                                      | 6                            | –                 |
| 664          | CIN3                                      | 6                            | –                 |
| 665          | CIN3                                      | 7                            | –                 |
| 669          | CIN2                                      | 5                            | 3800C > G         |
| 672          | CIN2                                      | 12                           | –                 |
| 680          | CIN3                                      | 6                            | –                 |
| 681          | CIN2                                      | 6                            | –                 |
| 834          | CIN3                                      | 8                            | –                 |
| 872          | CIN2                                      | 12                           | 3375C > T, 3575C > T, 5306G > T |

Analysis was performed on HPV16 positive cervical scrapes for both baseline CIN2/3 and rCIN2/3.

2. Materials and methods

2.1. Sample selection

Cervical scrapes from women (aged 18 + years) with CIN2/3 derived from a multicenter study (SIMONATH) which has been described earlier [11,12] and who were scheduled for LLETZ (Large Loop Excision of the Transformation Zone) treatment of CIN2/3 were included. Baseline scrapes were obtained between two weeks before up to immediately before LLETZ. In addition, preceding scrapes from women with a CIN2/3 found in the LLETZ material, without an additional biopsy could also be included. An additional scrape was taken from patients prior to treatment. For this study, only baseline CIN2/3 and rCIN2/3 scrapes were tested. Women treated for HPV16 positive baseline CIN2/3, and with HPV16 positive rCIN2/3 at six or 12-month follow-up were selected. Based on sample availability, a total of 14 HPV16 positive scrape pairs were tested, corresponding to 14 baseline CIN2/3 and 14 rCIN2/3 (6 rCIN2, 8 rCIN3), as shown in Table 1. The SIMONATH study was approved by the Medical Ethical Committee (METC) of VUmc (2009/285) and was registered in the Dutch Trial Registry (NTR1964).

2.2. HPV DNA detection

HPV detection and genotyping have been described previously for this study [11,12]. In short, total DNA was isolated from cervical scrapes using the Microlab start platform (Hamilton Robotics, Switzerland) with magnetic beads (Macherey-Nagel, Germany) according to the manufacturer’s protocol. HPV DNA was amplified using the GP5+/6 + PCR, followed by detection via enzyme immunoassay readout and genotyping via an in-house reverse line blot [13].

2.3. HPV16 whole genome amplification

Complete HPV16 genomes were amplified in ten fragments from selected samples using primers displayed in Table 2. PCR’s were performed using AmpliTaq Gold (Thermo Fisher Scientific, United States) according to the manufacturer’s protocol. Cycling conditions consisted of an initial incubation of 15 min at 95°, followed by 38 cycles of alternating 95° for 15 s, 55° for 30 s and 72° for 90 s, followed by a final elongation step at 72° for 10 min. Amplicon integrity was checked using the Lonza FlashGel (Lonza, Switzerland) system. If no product was observed, it was assumed the PCR performed at too little efficiency to observe on gel. Sample dsDNA concentrations were quantitated using the Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific, United States) according to the manufacturer’s protocol to facilitate equimolar pooling of PCR products.

2.4. Illumina NGS analysis

Samples were submitted to BaseClear for Illumina HiSeq PE125 sequencing. Raw sequencing data was subjected to trimmomatic 0.36 for quality and adapter trimming (SLIDINGWINDOW:5:25; MINLEN:35), followed by FastQC 0.11.6 and MultiQc 1.3 for quality checking. Trimmed sequences were assembled to a HPV16 (K02718) reference genome using Bowtie2 2.3.4. Assembly files (.sam) were converted to bam and indexed using samtools v1.6. Consensus sequences were extracted from bam files using samtools and seqtk 1.2. Variant calling files (vcf) were generated using Lofreq to assess the presence of variants at heterogeneous positions. The cutoff for minority variants was set at 0.5%. Finally, bed files were extracted using bedtools 2.27.1 to facilitate the generation of coverage plots in R.

2.5. Alignment and phylogeny

Consensus sequences were aligned using MUSCLE 3.8.1551. Maximum likelihood phylogenetic inference was performed using IQ-tree 1.5.5. The model finder option (-m MP) was used to identify the best fit model for this study, resulting in the HKY + F + I model. The final alignment was bootstrapped using IQ-tree’s ultrafast bootstrapping option (-bb 1000). Alignments were visualized using FigTree 1.4.3.

3. Results

3.1. Sample selection, amplification and sequencing

For this study, fourteen women treated for HPV16 positive CIN2/3, and with HPV16 positive rCIN2/3 at follow-up were selected (Table 1). Amplification of the whole HPV viral genome via overlapping PCR fragments was successful in all cervical scrapes. Subsequent ultra-deep sequencing of the cervical scrapes resulted in very high genome-wide coverage (Fig. S1), with a pooled average coverage per genome position of 112,287 (median: 104,130, minimum: 2045, maximum: 250,000), allowing for reliable assessment of infection variants.

3.2. Sequence comparison and characterization of nucleotide changes

Comparison of ultra-deep sequencing results of all fourteen paired samples showed (near) identical consensus variants at baseline and at recurrent disease, as shown in a maximum likelihood plot of the consensus sequence data in Fig. 1. Out of fourteen infections included in this study, ten are identical at the consensus level for both baseline and recurrent disease. In three patients, a single nucleotide polymorphism (SNP) was found at recurrent disease, at nucleotides 3723 and twice at 3800, respectively. In the remaining patient, three SNPs were detected at recurrent disease, at nucleotides 3375, 3575 and 5306. The sequence variations are listed in Table 1. A comparison of variant sequence, variant counts at each variable position and trinucleotide context, is presented in Table 3.

4. Discussion

In this study, we have investigated HPV16 variants in paired cervical scrapes of baseline CIN2/3 and rCIN2/3 at six or 12-month follow-up. Consensus variant analysis suggested that baseline CIN2/3 and rCIN2/3 are (near) identical in all cases. From a clinical perspective, this implies that the infection causing the initial CIN2/3 lesion was either not completely removed during treatment and resulted in a
recurrent lesion, or was caused by a novel infection with a (nearly) identical HPV16 variant. Our findings imply that in clinical practice, conventional PCR and genotyping is sufficient to detect type switches, since in the case of rCIN with the same HPV type as baseline CIN, both are likely caused by the same HPV variant.

Our comparison of consensus sequences showed that 10 out of 14 patients have identical HPV16 consensus genomes at baseline CIN2/3 and rCIN2/3. For these patients, the most parsimonious explanation is that the infection causing the initial CIN2/3 lesion also caused the rCIN2/3 lesion. The remaining four patients had consensus sequences that were nearly identical (1 and 3 nucleotide differences).

Previous studies have suggested that whole genome sequences differing ≥2 nucleotides could be considered unique variants, while sequences differing < 2 nucleotides cannot be reliably discriminated from each other [14]. In two patients, the rCIN2/3 positive HPV16 infection showed a one nucleotide difference compared to the HPV16 infection identified at baseline CIN2/3. Two more infections were found in which CIN2/3 and rCIN2/3 differed by two or three nucleotides. Considering the conservation of the HPV genome over time and the diversity of variants circulating in populations, the variants in the rCIN2/3 lesions most likely originated from the variants causing the initial CIN2/3. By definition, some, or all of these infections could be reinfections with unique variants, however, considering the plethora of different HPV16 variants circulating in populations [14,15] it is improbable that one would be repeatedly infected with so closely related variants.

Assessment of variants at the variable positions showed that in five out six cases, the consensus nucleotide at rCIN was already present as a minority at baseline CIN. In addition, five out of six SNPs are possible apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) mutations (C > G and C > T in a tCn trinucleotide motif) [16]. APOBEC mutations are suggested to accumulate naturally as a host response against viruses, and have been shown to occur on the HPV genome [14,17,18]. This could be particularly relevant for the patient with three nucleotide changes between CIN2/3 and rCIN2/3 (872), as all three changes are possible APOBEC mutations. Combined, these findings suggest that the variable nucleotides originated as mutation events, but the effect of treatment on their prevalence cannot be assessed, since this study lacks the appropriate controls and power for such an analysis.

**Table 2**

Primer sequences used in this study with references of origin. Primers developed for this study are marked with *.

| Forward    | Sequence 5′-3′                | Reference | Reverse    | Sequence 5′-3′                | Reference |
|------------|-------------------------------|-----------|------------|-------------------------------|-----------|
| F7869      | GGTAACATTTGACAGCAAC           | *         | R1312      | ACGATTGTGTTCGTTGATGGGC        | [14]      |
| F6835      | CTGTGCAAAATAACCTTAAGGC        | [14]      | R162       | GCACGTGGTGATCATGATGG           | [14]      |
| F5492      | TATAACTGACCAAGCTCCTCA         | [15]      | R6599      | TTATTTGTCGCCGTTGTC            | [16]      |
| F6201      | GAAACCTGCGGCGAAGGATGC         | [17]      | R6890      | GAATTCATAGAATGATGATG ATGTC    | [14]      |
| F3701      | CAGCTCAGTGGGATGGTACG          | [18]      | R5024      | AAGCAGGTCTACACACTTAA          | [14]      |
| F4930      | AACTAGTACGACACAACCAAC         | [14]      | R5725      | GCTGACAGATTCATCACTGC          | [17]      |
| F2529      | CAAACAAAGCTAGCTCCGAGG         | [14]      | R3551      | GTCACAGTCTGATCTGTC            | [14]      |
| F3387      | GTGGTACTATTAGTCCTCACCA        | [14]      | R4321      | ATGACAGTCTTGTGTCAG            | [14]      |
| F901       | ACGGGATCATGTAATGGATGG         | *         | R1780      | ATGACGATGATGAGACAC           | [16]      |
| F1832      | CAATGCTATGATGATGACCC          | [14]      | R2915      | ATGAGTCTATGATGATGAGG          | [14]      |

![Fig. 1](image_url)

**Fig. 1.** Maximum likelihood tree of consensus sequences obtained in this study. Baseline (B) and follow-up (G1/2) samples are shown to cluster close or identical to each other. X-axis shows genetic distance between samples.
Overall, most variations were observed in the E2 gene. The SNP at position 3375 is in the E2 hinge region, which is hypervariable between HPV types, and is not clearly associated with any function [19]. Position 3575 has been described as an integration site of HPV16 [20], although it is unknown how a nucleotide shift at this position affects this. Variations at positions 3723 and 3800 lead to changes in the DNA 

Table 3

| Sample | Nucleotide position | Total coverage | Major nucleotide | Coverage | Minor nucleotide | Coverage | Trinucleotide context |
|--------|--------------------|----------------|------------------|----------|------------------|----------|----------------------|
| 10B    | 3723               | 193,552        | T                | 169,840  | G                | 23,467   | gTg                  |
| 10G1   | 3723               | 167,524        | G                | 167,242  | –                | –        | –                    |
| 422B   | 3800               | 163,308        | C                | 146,166  | G                | 16,826   | tTa                  |
| 422G2  | 3800               | 153,665        | G                | 146,319  | C                | 7123     | tGa                  |
| 669B   | 3800               | 102,591        | C                | 102,279  | –                | –        | –                    |
| 669G1  | 3800               | 138,622        | G                | 131,277  | C                | 7121     | tGa                  |
| 872B   | 3375               | 99,262         | C                | 56,321   | T                | 42,874   | tCc                  |
| 872G1  | 3375               | 105,095        | T                | 93,927   | C                | 11,124   | tCc                  |
| 872B   | 3576               | 92,831         | C                | 50,679   | T                | 42,041   | tCa                  |
| 872G1  | 3576               | 106,969        | C                | 96,364   | C                | 10,557   | tGa                  |
| 872B   | 5306               | 111,061        | C                | 59,651   | T                | 50,363   | tCa                  |
| 872G1  | 5306               | 118,822        | T                | 98,347   | C                | 20,445   | tGa                  |

Conflicts of interest

RDMS and CJLMM are minority shareholders of Self-Screen B.V., a spin-off company of VUMc, of which CJLMM is part-time director since September 2017. Self-screen holds patents on methylation markers and HPV detection. CJLMM has received speakers’ fee from Qiagen, SPMSD/Merck, served occasionally on the scientific advisory board (expert meeting) of Qiagen, SPMSD/Merck and GSK. CJLMM has a very small number of shares in Qiagen, and was minority shareholder of Diassay until April 2016. CJLMM has been co-investigator on a SPMSD sponsored trial, for which his institute received research funding. PvdW and AJK have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pvr.2019.04.008.

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