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Sequencing detects human papillomavirus in some apparently HPV-negative invasive cervical cancers

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Abstract

Introduction. Cervical cancer is caused by human papillomavirus (HPV), but some cases may test HPV-negative. We previously tested 2850 Swedish cases and found that 394/2850 (13.8%) cases tested HPV DNA-negative by PCR. Sequencing is the most thorough method to assess HPV status.

Aim. We wished to assess whether deep sequencing might detect HPV sequences among these HPV-negative cervical cancer specimens, and to increase the likelihood of detecting transcriptionally active infections.

Methodology. Out of the 2850 cancer cases, we sequenced a random sample of 92 HPV PCR-negative cervical cancers and 34 HPV PCR-positive cervical cancers. Four pools of blank blocks were sequenced as negative controls. To enrich for mRNA – a hallmark of active viral infection – the samples were extracted, reverse-transcribed, rRNA-depleted and then sequenced using the NovaSeq 6000 system (Illumina, USA). High-quality reads were aligned to the human genome and non-human reads were queried against HPV proteins.

Results. We obtained a median of 23 million paired reads per sample. HPV was detected in 31/34 HPV PCR-positive cases. Among cases negative for HPV by PCR, 48/92 (52.2%) contained HPV sequences, with HPV33 being the most commonly detected type among these (14/48 cases, 29.2%). Comparison of the ratio of exon and intron sequences found that the sequenced material contained both DNA and RNA. Splice junctions were detected in 12 cases.

Conclusion. Apparently, some cervical cancers contain HPV that is difficult to detect by PCR. Sequencing may be a helpful tool for additional quality assurance for HPV testing methods.

INTRODUCTION

Human papillomavirus (HPV) is the major cause of cervical cancer. The usual methods to screen for HPV-associated lesion and cancers are based on PCR, usually targeting the *L1* gene, which is the most conserved gene within the HPV genome [1]. PCR methods are both efficient and sensitive, but are also biased to only detect HPVs that bind specifically to designed primers and probes. HPV types that diverge in their nucleotide sequences from the primers/probe sequences may escape amplification and will remain undetected [2]. For optimal cervical screening, it is essential to as far as possible limit the risk of false-negative tests. Previous studies have shown that cervical cancers testing HPV-negative by PCR constitute a biologically distinct subgroup, associated with symptomatic detection, late-stage diagnosis and worse prognosis [3].

With the dramatically decreased cost of sequencing, it is now easy to perform sequencing of samples and with bioinformatics detect all HPVs present in a sample, without prior knowledge of which types might be present [2, 4, 5]. Unbiased

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Abbreviations: ACTB, Actin beta; Bp, Base pairs; FFPE, Formalin-fixed, paraffin-embedded; HPV, Human papillomavirus; PaVE, Papillomavirus Episteme.

All the aligned, non-human sequences are available at the Sequence Read Archive (SRA) within the bio-project ID PRJNA563802 (https://www.ncbi.nlm.nih.gov/sra/PRJNA563802).

One supplementary figure is available with the online version of this article.

analysis of the nucleic acid sequences will detect any HPV type present in the sample, with no need to design primers and probes for previously known sequences. Sequencing of mRNA is of particular interest, as it will show if the HPV that is present has transcriptional activity. Viral oncogene transcription is essential for both initiation and maintenance of the malignant phenotype.

We wished to assess whether cervical cancer specimens testing HPV-negative by PCR may still contain HPV sequences that can be found by sequencing. Through the generation of cDNA prior to sequencing, we further aimed to increase the likelihood of detecting transcriptionally active infections.

METHODS

Sample material

Previously [6], we identified and requested formalin-fixed, paraffin-embedded (FFPE) blocks from all invasive cervical cancers in Sweden from 2002 to 2011 (*n*=4254). All obtained blocks (*n*=2932) were extracted with a xylene-free method and the nucleic acid extracts were stored at −20 °C [7].

A total of 2850/2932 cancers had adequate HPV typing results, with 394/2850 (13.8%) cases being 'apparently HPV-negative' after being tested for HPV DNA with both PCR with MGP primers targeting the *L1* gene, and real-time PCR with primers targeting the E7 gene for HPV16, and the E6 gene for HPV18 [6].

Out of these 394 cases, we included a randomly selected sub-set of cervical tumour specimens. We analysed a total of 136 samples, including 34 HPV-positive cervical specimens (L1 positive for PCR) used as positive controls, 92 'apparently HPV-negative' cervical specimens, 4 pools comprising all blank blocks from all tumour blocks as negative controls and 6 duplicates to evaluate the reproducibility of the study. Blank blocks from each case block, both positive and negative for HPV, had been picked out and pooled into four negative control pools.

Sequencing

All 136 samples (126 specimens, 6 duplicates and 4 blank blocks) were extracted [7], reverse-transcribed, ligated to individual adapters and rRNA-depleted using the SMARTer pico kit according to the manufacturer's protocol (Takara, USA). Libraries were validated, normalized to 2 nM and pooled before sequencing. Sequencing was performed using the NovaSeq 6000 system (Illumina, USA), 2×150 bp, in two different runs. The first sequencing run comprised 30 HPV-positive specimens, 33 cancer specimens that had been HPV-negative by PCR and 2 pools containing all blank blocks from these 63 specimens. Sequencing was performed on 2 S1 lanes aiming for 2000 million paired reads (30 M paired reads per sample). The second sequencing run comprised 5 HPV-positive specimens, 64 apparently HPV-negative cancer specimens and 2 pools containing all blank blocks from these 69 specimens. The six duplicates used for reproducibility testing were already included among these samples. Duplicates corresponded to one HPV16 PCR-positive specimen and 5 HPV PCR-negative specimens. Sequencing was performed on 1 S4 lane aiming for 2000–2500 million paired reads (30 M paired reads per sample). Pools containing blank blocks were made randomly.

Bioinformatic analysis

Indices, included in the Illumina adaptors, were used to assign raw sequence reads obtained from the NovaSeq 6000 (Illumina) platform to the originating samples. Reads were quality- and adaptor-trimmed with Trimmomatic [8]. All reads with a read length below 18 base pairs (bp) were discarded for further analysis and the first 3 nucleotides from every R2 read were trimmed, as indicated within the SMARTer pico kit used for library preparation. High-quality reads were screened against the human reference genome GRCh38 using NextGenMap [9] and human reads (reads mapping with >95% identity over 75% of their length) were filtered from the data set. Non-human reads were queried against all HPV protein sequences included in the PaVE database (Papillomavirus Episteme, accessed on 28 July 2019, including all protein sequences from HPV reference and non-reference genomes), using the open source software Diamond [10] BLASTX with default parameters and –top 1. Samples were considered positive for HPV if a minimum of five reads were detected for any HPV type with at least 90% identity. Samples presenting a co-infection of HPV types were subjected to manual investigation to confirm positivity for the corresponding genotypes in order to avoid false positivity due to both genotypes presenting close phylogeny.

Confirmation of HPVs with a low number of reads

Sequenced specimens that were positive with a low number of reads for an HPV type that had been present in another specimen at a high number of reads in the same sequencing run were selected for independent verification. Misclassification of index sequences, (particularly if the sequences are of low quality) may result in erroneous assignment of sequence reads to the wrong sample (so-called 'index hopping') [11]. Samples that presented <2% of the reads found in another specimen analysed in the same run were either confirmed with previous genotyping results or resequenced. Resequencing was performed using new libraries (the same protocol as described above) and sequencing with the NextSeq 500 system (Illumina, USA) at 151 paired-end cycles, according to manufacturer's instructions.

Analysis of DNA and cDNA amounts in the sequenced samples

Because the nucleic acid extraction method used is not specific for DNA or RNA and the reverse transcription method used to obtain cDNA does not contain any DNA depletion step, we wished to assess whether the sequences obtained were derived mostly from genomic DNA or from cDNA. The reads were mapped to introns and exons of the human reference protein coding gene, actin beta (ACTB). The presence of cDNA was also studied by analysing whether there were reads

Table 1. Characteristics of cervical tumour specimens

	HPV– (*n*=92)	HPV+ (*n*=34)	Total (*n*=126)
Age at cancer diagnosis			
Median (range)	61 (26–91)	57.5 (25–95)	60 (25–95)
<29	1 (1.1)	1 (2.9)	2 (1.6)
30–44	23 (25.0)	9 (26.5)	32 (25.4)
45–59	18 (19.6)	8 (23.5)	26 (20.6)
60–74	27 (29.3)	8 (23.5)	35 (27.8)
>74	23 (25.0)	8 (23.5)	31 (24.6)
FIGO stage			
IA	12 (13.0)	4 (11.8)	16 (12.7)
IB	25 (27.2)	15 (44.1)	40 (31.7)
II	17 (18.5)	8 (23.5)	25 (19.8)
III+	38 (41.3)	7 (20.6)	45 (35.7)
Cancer types*			
Squamous cell carcinoma	69 (75.0)	29 (85.3)	98 (77.8)
Adenocarcinoma	15 (16.3)	3 (8.8)	18 (14.3)
Adenosquamous cell carcinoma	4 (4.3)	1 (2.9)	5 (4.0)
Other types†	4 (4.3)	1 (2.9)	5 (4.0)

The numbers in parentheses show the percentage of specimens, calculated as the number for each level divided by the total number of HPV– and HPV+ cases, respectively (e.g. 13% of specimens that were HPV-negative were classified as FIGO stage IA).

*Cancers that were not primary, invasive, epithelial, or recurrent cases were excluded.

†Other cancer types, including small cell carcinoma, large cell carcinoma, neuroendocrine carcinoma and undifferentiated cell carcinoma.

that contained sequences known to represent splice junction sequences for viral mRNAs.

RESULTS

One hundred and twenty-six cervical cancer specimens (34 HPV-positive and 92 apparently HPV-negative) were sequenced using NovaSeq 6000 (Illumina, USA) and analysed bioinformatically for HPV detection. The median age at diagnosis of the cases was 60 years (range 25–95 years); most women were aged 43–74 at diagnosis. Seventy-eight per cent of the cases had squamous cell carcinoma, 14% had adenocarcinoma and 8% were diagnosed with rarer histological types, such as adenosquamous carcinoma. Compared to cases originally positive by HPV PCR, cases originally HPV-negative were generally older and more often presented with a higher stage (III+) at diagnosis (Table 1).

The sequencing generated high-quality sequencing data, with a median of 1800 M paired reads per run and 23 M paired reads per sample. Most reads present in the specimens (an average of 95% of the total reads) were human sequences.

We found HPV sequences in 31/34 specimens originally designated positive by HPV PCR, with total concordance regarding the HPV type detected in 29/31 specimens (Table 2). Partial concordance was detected in one specimen. This cervical tumour had been genotyped as HPV16, but sequencing found both HPV16 and HPV40 sequences. In 3/34 HPV-positive specimens we did not find HPV sequences of the type detected by PCR. One specimen that had been genotyped as HPV16 was found to contain sequences from both HPV33 and HPV73. Confirmation of HPV co-infections in HPV-positive specimens (*n*=3) was performed by blasting and visualizing reads manually (Table 2).

We further found HPV sequences in 48/92 specimens originally deemed negative by HPV PCR (52.2%). Almost all positive specimens contained sequences from only a single HPV type (93.8%, 45/48). HPV33 and 73 were the most common types detected (14/48 and 8/48, respectively) (Table 3). As 13 of the 17 different HPV types detected should have been detected by the PCR-based method, we analysed the coverage of HPV reads within the MGP region in the *L1* gene (the PCR region targeted for genotyping) as well as possible mismatches with primers and probes (Table 3). Twenty-nine specimens (29/48, 60.4%) showed HPV genotypes with no reads at all covering the MGP region. Two HPV types from the 19 specimens that did contain reads in the MGP region instead presented nucleotide substitutions when their sequence was compared with the respective HPV reference clone used for MGP optimization [12] and the probe (Table 3).

Sequences from several HPV types were detected in three samples that contained sequences from HPV31/58, HPV31/62 and HPV4/53/203, respectively. Confirmation of the presence of multiple different HPV types within the same specimen was performed by blasting and visualizing reads manually. None of the blank blocks used as negative controls presented any reads belonging to the HPV genotypes present within the specimens.

Six specimens were extracted and subjected to library preparation and sequencing twice (so-called duplicates) to assess reproducibility of the study. These included one HPV16 PCR-positive specimen and five HPV PCR-negative specimens. Novaseq confirmed HPV16 positivity in the corresponding HPV16 PCR-positive specimen as well as HPV negativity in 4/5 HPV PCR-negative specimens. One specimen that had previously been classified as HPV PCR-negative was deemed positive in Novaseq sequencing (HPV33). All six duplicates presented the same result when resequenced.

Confirmation of HPVs with a low number of reads

To avoid false-positive results, in addition to checking whether any HPV type was found among all specimens in any of the four negative controls (blank blocks from all cancer cases),

Table 2. HPV sequencing detection for HPV-positive cervical tumour specimens

Sample ID	Genotyping HPV	Sequencing HPV (reads)	Sample ID	Genotyping HPV	Sequencing HPV (reads)
A7	16	16 (108)	A6	18	18 (73)
A17	16	16 (622)	A12	18	18 (54)
A18	16	16 (16)	A13	18	18 (10)
A21	16	16 (1534)	A47	18	18 (434)
A26	16	16 (2264)	A59	18	18 (16)
A28	16	16 (111)	B37	18	18 (82)
A34	16	16 (274)	A41	33	33 (1313)
A36	16	16 (12)	A53	33	33 (350)
A37	16	16 (729)	A58	33	33 (1450)
A43	16	16 (147)	A1	45	45 (181)
A44	16	16 (7168)	A9	45	45 (44)
A45	16	16 (389)	A50	16,33	16 (6), 33 (3219)
A62	16	16 (2270)	A60[a]	16	16 (804), 40 (169)
B1	16	16 (585)	A48[b]	16	33 (8), 73 (86)
B57	16	16 (18098)	A33[b]	16	Negative
A42	58	58 (8450)	B40[b]	16	Negative
A46	58	58 (120)	A22[b]	45	Negative

The numbers in parentheses showthe total number of reads detected. Genotyping HPV results correspond to samples being HPV L1-positive by PCR. The letters A and B for sample IDs clarify which samples were subjected to the first and the second sequencing run, respectively. *a*, specimens with partial concordance. *b*, specimens with discordant results.

we considered whether the apparently PCR HPV-negative specimens that were deemed positive by sequencing and presented a low number of reads might have been mistakenly classified as HPV-positive due to a phenomenon known as 'index hopping', where similar indexed adapters are misread during multiplexed sequencing, resulting in some sequences being assigned to the wrong sample.

Samples that presented <2% of the HPV reads of another sample analysed in the same run (n=12/126; 8 HPV16, 3 HPV33 and 1 specimen with HPV58) were analysed further for positivity verification (Table 4).

Genotyping results confirmed HPV16 and HPV58 positivity in 5/8 and 1/1 sample(s), respectively. We resequenced the other three HPV16-positive specimens and three HPV33 specimens. Resequencing with NextSeq included one positive control, one negative control and 5/6 specimens (one HPV16 specimen could not be resequenced because there was not enough sample material left). HPV33 presence was confirmed in 3/3 analysed specimens. HPV16, however, was not detectable in 1/3 specimens and the other resequenced specimen showed the presence of HPV33 (3052 reads) but no trace of HPV16.

Analysis of DNA and cDNA amounts

Fragment analysis showed large gDNA molecules after extraction and the visualization of sequencing reads mapping the introns of the ACTB gene (Fig. S1, available in the online version of this article) confirmed the presence of DNA. The median exon/intron ratio – calculated as (reads covering the exons/total nucleotide length of exons)/(reads covering the introns/total nucleotide length of introns) – was 1.49 (max: 9.31; min: 0.59). In order to confirm existence of cDNA, we looked for known splice junctions belonging to spliced HPV transcripts and detected reads with these splice junctions in 12 specimens (Table 5). Negative blank blocks showed no reads in the ACTB gene, as expected.

DISCUSSION

Cervical cancer is caused by HPV, but a proportion of cervical carcinomas may still test negative for HPV in PCR [3, 13]. Besides true negativity, false negativity may occur because of either (i) misclassification of the tumour (in particular endometrial cancers or metastases of other cancers may be misclassified as cervical cancers), (ii) specimen inadequacy, (iii) detection method insensitivity, (iv) integration events causing disruption of HPV and partial loss of sequences, (v)

Table 3. HPV detection in 'apparently HPV-negative' cervical cancer specimens (*n*=48)

HPV type	n	MGP region coverage	Observations
4	1	NA	
16	3	0/3	
30	1	0/1	
31	7	1/7	Same as reference genome
33	14	13/14	Same as reference genome
35	1	1/1	Presents 2 substitutions at the probe
39	1	0/1	
45	3	0/3	
53	1	0/1	
54	1	0/1	
56	2	0/2	
58	4	¼	Same as reference genome
59	2	0/2	
62	1	NA	
73	8	3/8	1 specimen shows 3 substitutions at the probe.
203	1	NA	
211	1	NA	
Total	48	19/48	

The MGP region coverage column shows how many specimens presented reads from the corresponding genotype covering the MGP region. Genotypes presented in bold are genotypes that had specific probes when genotyping. Note that the total number of samples is 48 (not 52) due to 3 specimens presenting multiple HPV types (HPV31/58, HPV31/62 and HPV4/53/203).
n, number of specimens where the genotype was found. NA, not applicable.

sequence variability in the regions of the virus targeted by the detection methods and (vi) the existence of novel HPV types that are not detectable by the method used [14]. We had carefully re-reviewed all cancer slides to confirm diagnosis and assessed specimen adequacy by performing beta-globin detection [6], thus options (i) and (ii) above could not have caused false-negative observations. By performing an unbiased (not based on methods requiring prior knowledge of the sequences being looked for) sequencing, we aimed to increase sensitivity and analyse possible biological reasons for why these cervical cancers had tested HPV-negative.

The nature of our specimens (FFPE) was not ideal to perform RNA sequencing, and the extraction method used was not specifically optimized to extract both DNA and RNA [7]. Pilot studies were originally performed with DNA sequencing for these samples (Nextera library preparation,

Table 4. HPVs detected and reads

	Total samples	Maximum no. of reads	Minimum no. of reads	Samples with <2% of maximum reads
HPV4	1	6	6	0
HPV16	20	18098	6	8
HPV18	6	434	10	0
HPV30	1	14	14	0
HPV31	7	105	8	0
HPV33	19	3219	8	3
HPV35	1	738	738	0
HPV39	1	28	28	0
HPV40	1	169	169	0
HPV45	5	516	14	0
HPV53	1	35	35	0
HPV54	1	272	272	0
HPV56	2	238	76	0
HPV58	6	8450	8	1
HPV59	2	8	6	0
HPV62	1	8	8	0
HPV73	9	561	18	0
HPV203	1	12	12	0
HPV211	1	6	6	0

Table 5. Spliced junctions detected in HPV reads

HPV (PCR)	HPV (sequencing)	Total HPV reads (sequencing)	Spliced transcripts detected
16	16	2270	E1^E4 (2 reads)
18	18	434	E6* (2 reads)
45	45	181	E6* (2 reads)
45	45	181	E6* (2 reads)
Negative	33	1148	E1^E4 (10 reads)
Negative	35	738	E1^E4 (2 reads)
Negative	33	302	E1^E4 (32 reads)
Negative	54	272	E1^E4 (7 reads)
Negative	33	54	E1^E4 (8 reads)
Negative	45	14	E6* (2 reads)
Negative	45	516	E6* (4 reads)
Negative	73	561	E6* (4 reads)

Each row corresponds to one specimen.

Rubicon) and the libraries did not reach the quality requirements (data not shown), and so we opted not to proceed solely with DNA-based sequencing. We chose to proceed with the SMARTer Pico Input library preparation protocol, which includes primers that amplify both RNA and gDNA if such are present in the sample. This was more successful and we were able to detect large-molecular-size nucleic acids, most probably corresponding to degraded gDNA. We do not know if the reads assigned to coding regions were from mRNA or DNA. However, the detection of reads with known viral splice junctions in 12 specimens is formal proof that at least some mRNA was present in these FFPE samples.

In summary, we report that HPV sequences exist in >52% cervical cancer specimens that had previously tested negative by sensitive HPV PCR. Some sequences were from HPV types that we had not tested for (HPV4, HPV62, HPV 203 and HPV211). Most (29/48) of the PCR-negative cervical cancers had no sequences present from the L1 region in the samples, which might explain why a PCR method targeting L1 failed to detect the HPV. Two samples showed sequence variability in the sequences targeted by primers or probes. For the remaining 17 cancer specimens, we cannot explain why the HPV was not detected. Our previously used method has a reported sensitivity of 50 international units of HPV16 and HPV18 and 500 genome equivalents for the other oncogenic HPV types [15], suggesting that the difference may be attributable to the higher sensitivity of the sequencing. Further studies are needed to understand why HPV may become difficult to detect in late-stage cancers and how this may affect the design and quality assurance of optimal tests for HPV-based cervical screening.

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Author contributions
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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Ethical approval was granted to perform histopathology review of the diagnostic slides and to collect all archival material from cervical cancer cases to perform genotyping (EPN-Dnr: 2012/1028/32). The Regional Ethical Review Board determined that, due to the population-based nature of the study, informed consent from study participants was not required (EPN-Dnr: 2011/1026-31/4).

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