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ORIGINAL PAPER

Detection of rare and possibly carcinogenic human papillomavirus genotypes as single infections in invasive cervical cancer

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Abstract

The contribution of carcinogenic human papillomavirus (HPV) types to the burden of cervical cancer has been well established. However, the role and contribution of phylogenetically related HPV genotypes and rare variants remains uncertain. In a recent global study of 8977 HPV-positive invasive cervical carcinomas (ICCs), the genotype remained unidentified in 3.7% by the HPV SPF_{10} PCR–DEIA–$LiPA_{25}$ (version 1) algorithm. The 331 ICC specimens with unknown genotype were analysed by a novel sequence methodology, using multiple selected short regions in L1. This demonstrated HPV genotypes that have infrequently or never been detected in ICC, ie HPV26, 30, 61, 67, 68, 69, 73 and 82, and rare variants of HPV16, 18, 26, 30, 34, 39, 56, 67, 68, 69, 82 and 91. These are not identified individually by $LiPA_{25}$ and only to some extent by other HPV genotyping assays. Most identified genotypes have a close phylogenetic relationship with established carcinogenic HPVs and have been classified as possibly carcinogenic by IARC. Except for HPV85, all genotypes in α-species 5, 6, 7, 9 and 11 were encountered as single infections in ICCs. These species of established and possibly carcinogenic HPV types form an evolutionary clade. We have shown that the possibly carcinogenic types were detected only in squamous cell carcinomas, which were often keratinizing and diagnosed at a relatively higher mean age (55.3 years) than those associated with established carcinogenic types (50.9 years). The individual frequency of the possibly carcinogenic types in ICCs is low, but together they are associated with 2.25% of the 8338 included ICCs with a single HPV type. This fraction is greater than seven of the established carcinogenic types individually. This study provides evidence that possibly carcinogenic HPV types occur as single infections in invasive cervical cancer, strengthening the circumstantial evidence of a carcinogenic role.

Keywords: human papillomavirus; carcinogenic; oncogenic; high-risk; cervical cancer; HPV26; HPV30; HPV67; HPV73; HPV82

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Introduction

The human papillomavirus (HPV) genotype distribution was recently determined in 10 575 globally collected invasive cervical carcinoma (ICC) specimens [1]. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were analysed by the highly sensitive HPV SPF_{10} PCR–DEIA–$LiPA_{25}$ (version 1) algorithm [2,3]. HPV DNA was detected in 8977 of 10 575 (84.9%) ICC cases. A previous paper reported HPV DNA detection in 99.7% of ICC cases [4], suggesting a strong causal association between ICC and HPV. However, this was based on frozen materials, in which HPV is more easily detected, selected from a limited range of sources. In the routinely collected specimens used in our study, the quality of the DNA is expected

to be variable even for detection of HPV by a highly sensitive PCR.

Among 8977 HPV-positive cases, genotypes were recognized in 96.3% and the HPV genotype remained unidentified by $LiPA_{25}$ in 331 (3.7%), suggesting the presence of a rare, untargeted type or variant. Other widely used genotyping assays, eg Reverse Line Blot [5] or Linear Array HPV Genotyping Test (Roche Diagnostics, Almere, The Netherlands), are also limited in the genotypes targeted. Moreover, these assays are not suitable for FFPE specimens. Therefore, a novel methodology was developed for genotyping ICC cases with unknown HPV. This technique used broad-spectrum amplification of multiple, short fragments of HPV DNA in the L1 open reading frame, followed by sequence analysis. PCR primer sets that amplify a small region are less vulnerable to nucleic acid fragmentation and irreversible cross-linking in FFPE tissue specimens [6].

Recently, the IARC has classified HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 as carcinogenic or probably carcinogenic (Groups 1 and 2A), based on epidemiological, phylogenetic and limited mechanistic evidence [7,8]. Other HPV types have a low prevalence in ICC and their carcinogenic role remains uncertain. A number of these HPVs (eg HPV6 and 11) have been classified as 'low-risk' by a case-control study [9]. Their prevalence in healthy women is much higher than in women with ICC. The association of other anogenital HPVs with ICC remained undetermined because they are rarely targeted by HPV genotyping tests.

All low-risk types except HPV70 have been classified as non-carcinogenic (Group 3) because they do not share a strong phylogenetic relationship with established carcinogenic HPVs [10]. HPVs with undetermined or uncertain epidemiological risk but a close phylogenetic relationship with established carcinogenic HPVs have been classified as possibly carcinogenic by IARC (Group 2B), eg HPV26, 30, 34, 53, 66, 67, 69, 70, 73, 82, and 85. Together, HPVs from α-species 5, 6, 7, 9 and 11 form a carcinogenic or high-risk evolutionary clade. Possibly carcinogenic types were found in ICCs as part of a global typing study [1]. In this paper we describe the new sequence methodology used to identify these types in FFPE samples. This paper additionally reports the phylogenetic classification of rare, possibly carcinogenic, HPVs and the detailed pathological features and age distribution of ICC associated with these types as single infections from the largest global collection of ICC so far. We found that possibly carcinogenic types were usually associated with typical, often keratinizing, squamous cell carcinoma diagnosed at a higher mean age than established carcinogenic types.

Materials and methods

Collection and processing of ICC tissue specimens

From a retrospective, global study of 10 575 FFPE specimens of ICC, 331 HPV DNA-positive cases with unidentified HPV types were selected. The specimens were obtained between 1949 and 2009. The specimens and strategies for HPV genotype identification are depicted in Figure 1. The collection of tissue blocks, 'sandwich' cutting for PCR, histopathological assessment and DNA extraction by proteinase K treatment is described by de Sanjose *et al* [1].

The local and Institut Català d'Oncologia ethics committees approved all protocols. The study was supervised by an international steering committee.

Genotyping by SPF_{10} PCR–DEIA–$LiPA_{25}$

Proteinase K-treated DNA was diluted 10-fold prior to performing HPV SPF_{10} PCR–DEIA–$LiPA_{25}$, version 1 (Labo Biomedical Products, Rijswijk, The Netherlands). SPF_{10} primers amplify more than 54 HPV genotypes for general HPV detection by DEIA and $LiPA_{25}$ can genotype 25 (possibly) carcinogenic and non-carcinogenic HPVs [2,3,11].

DEIA demonstrated HPV positivity in 8977 cases. One or more genotypes were recognized in 8646 cases (96.3%) by $LiPA_{25}$. The remaining 331 HPV-positive cases (3.7%) represent two patterns of results. In 198 (2.2%), probe lines of $LiPA_{25}$ did not identify an HPV type. $LiPA_{25}$-negative cases, confirmed by repeat testing, were analysed by a newly developed sequence methodology in three regions of L1. In 133 ICC cases (1.5%), the probe line pattern of $LiPA_{25}$ was inconclusive for the presence of HPV18, 39, 68 or 73 [3]. Sequence analysis of only one region was sufficient to differentiate between these types.

Sequencing of ICCs of unknown type

Strategies for sequence analysis

Three distinct sequence primer sets were developed to amplify short fragments of viral DNA in the L1 open reading frame (Figure 2). These PCRs are specifically designed for FFPE specimens that have a high amount of fragmented DNA and irreversible cross-linking. The first broad-spectrum sequence PCR targets the 65 bp SPF_{10} region. The SPF_{10} sequence information explains why $LiPA_{25}$ probes were not able to hybridize. Mismatches with the $LiPA_{25}$ probes could indicate presence of untargeted types or variants.

Two larger sequence PCRs, primer sets I and II, were designed to provide more sequence information for HPV genotype or variant identification. These fragments encompass the SPF_{10} region for confirmation of the initial SPF_{10} sequence result, but have the disadvantage of decreased amplification efficiency by targeting a longer fragment. Primer set I amplifies a 184 bp fragment, covering the SPF_{10} region

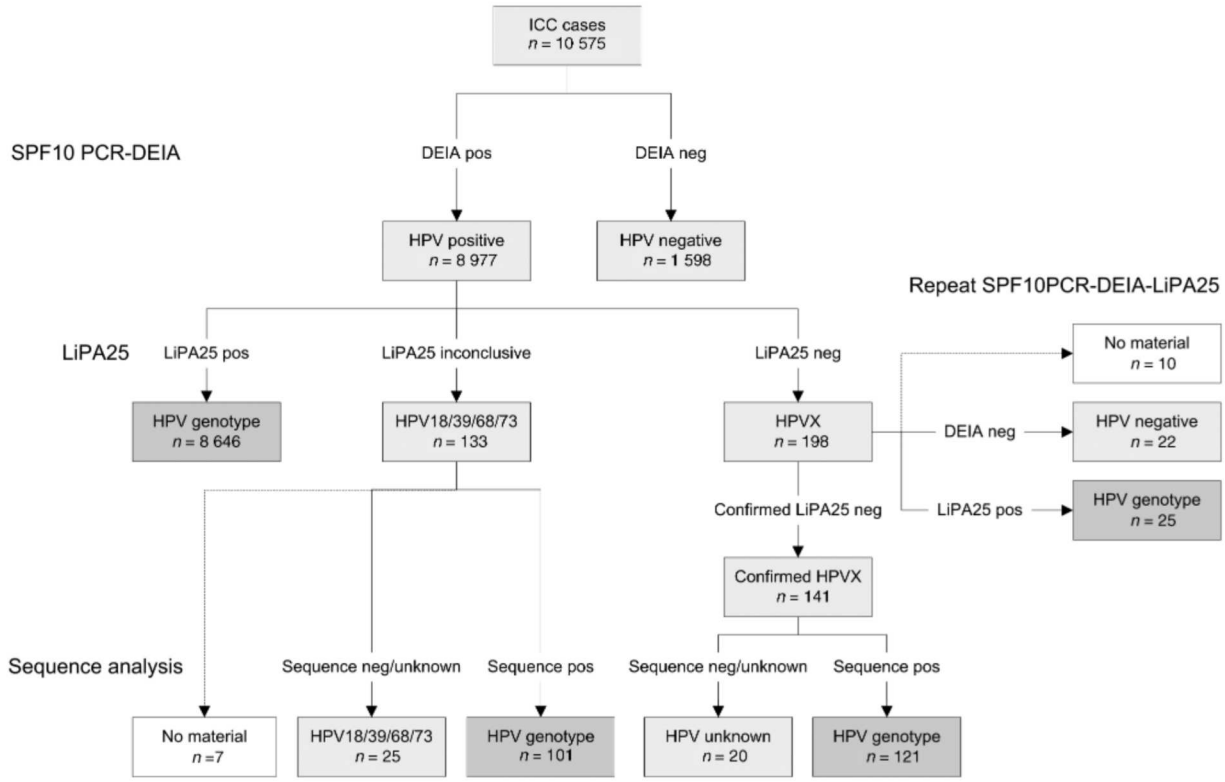

Figure 1. Schematic overview of the intake of clinical samples and strategies to determine the HPV genotype.

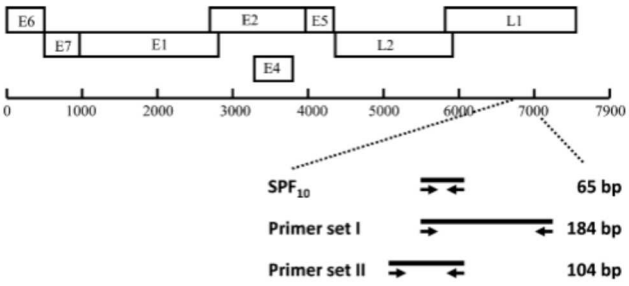

Figure 2. Schematic overview of the HPV16 genome and the three separate primer sets in the L1 ORF for HPV sequencing. Adapted from Kleter *et al* [3].

as well as a downstream flanking region. Primer set II generates a 104 bp product, containing SPF_{10} and an upstream adjacent region. The sequence targeted by primer sets I and II combined covers 223 bp of L1.

Sequence analysis by SPF_{10}

Sequence analysis of the SPF_{10} regions was carried out as described previously [1]. The 22 bp SPF_{10} inner primer region is too short for direct sequence analysis. Therefore, SPF_{10} PCR products were re-amplified with SPF_{10} primers extended with SP6 and T7 linker sequences. The presence of the 105 bp re-amplification product was verified by a 2.2% FlashGel (Lonza, Basel, Switzerland). This amplimer was purified from unconsumed primers by ExoSAP-IT (USB, Staufen, Germany). Finally, SP6 and T7 primers were used for bidirectional sequence analysis.

Sequence analysis by primer sets I and II

Primer set I comprises forward primers aimed at positions 6582–6601 and reverse primers aimed at positions 6741–6765 (numbering according to HPV16 Accession No. K02718). Primer set II comprises forward primers aimed at positions 6543–6565 and reverse primers aimed at positions 6624–6646.

Briefly, both PCRs were performed in a final reaction volume of 50 µl, containing 10 µl 10-fold diluted isolated DNA, 1× GeneAmp PCR buffer II (Applied Biosystems), 0.2 mM concentrations of deoxynucleoside triphosphates (GE Healthcare/Isogen Life Science, De Meern, The Netherlands), 7.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 3 mM $MgCl_2$ (primer set II) or 3.5 mM $MgCl_2$ (primer set I) (Applied Biosystems).

Amplification by primer sets I and II was performed by a 9 min preheating step at 94 °C, followed by 40 cycles of amplification comprising 30 s at 94 °C, 45 s at either 38 °C or 52 °C, and 45 s at 72 °C. A final elongation step of 5 min at 72 °C ended the PCR.

The presence of amplimers generated with primer sets I and II was verified on a 2.2% FlashGel. Subsequently, amplimers were purified with ExoSAP-IT. Bidirectional DNA sequencing was performed using the BigDye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI3100 Avant Genetic Analyser (Applied Biosystems), according to the manufacturer's instructions.

When non-specific bands were observed on FlashGel using primer sets I and II, all remaining PCR product

was loaded and separated in a conventional, home-made 2% agarose gel. Fragments of the correct size were excised from the gel and purified using the QIA quick gel extraction kit (Qiagen, Hilden, Germany), according to the kit manual instructions, and eluted in 35 µl elution buffer prior to sequence analysis.

Pathology review

The histological diagnosis of ICC cases had been initially performed at the reference pathology laboratory of the Institut Català d'Oncologia, as described previously [1]. All ICC cases with a single possibly carcinogenic HPV type were squamous cell carcinomas ($n = 185$), apart from two adenocarcinomas and one adenosquamous carcinoma. All available cases were included in this study and reviewed by an additional pathologist, who classified these according to the World Health Organization (WHO) classification [12]. Only haematoxylin and eosin (H&E)-stained slides of the squamous cell carcinomas were available, and were subclassified as keratinizing, papillary, warty or basaloid.

Statistical analysis

Simple frequencies and percentages are described for qualitative variables. For age at diagnosis, we calculated measures of central tendency and dispersion [mean, median, standard deviation (SD) and rank]. To assess differences between mean ages at diagnosis among groups of HPVs, we performed Student's *t*-test for IARC group comparison. One-way ANOVA with Tamhane's test for *post hoc* multiple comparisons was used between α-species. Statistical significance was set at $p = 0.05$. For each type we calculated the ratio of its mean age and global mean age, including 95% confidence intervals.

Results

Sequencing of ICCs with negative $LiPA_{25}$ result

Confirmation of $LiPA_{25}$-negative cases by repeat testing

The initial DEIA-positive, $LiPA_{25}$-negative finding was confirmed in 141/188 (75.0%) cases with available material. The $LiPA_{25}$ algorithm identified a genotype in 25/188 ICCs (13.3%), while 22/188 specimens (11.7%) were HPV-negative.

Sequence analysis by SPF_{10} and primer sets I and II

The 141 SPF_{10} amplimers of confirmed $LiPA_{25}$-negative cases were sequenced. The SPF_{10} sequence was successfully determined in 125/141 cases (88.7%). Non-interpretable sequences were obtained in 16/141 samples (11.3%).

121/125 sequences (96.8%) did not match the probes required for type-recognition by $LiPA_{25}$. Sequence analysis by primer set I was performed on these 121 cases and provided a positive result in 47/121 cases (38.8%). Sequence information by SPF_{10}, and if available by primer set I, was not considered sufficient for 54/121 ICC specimens. Sequence analysis by primer set II was performed on these 54 cases and yielded interpretable sequences for 40/54 specimens (74.1%).

Comparison with GenBank sequences

SPF_{10} and available flanking sequences were compared to those already reported in the GenBank database, using BLAST software [13]. 71/121 (58.7%) submitted sequences were identical to a classified reference HPV genotype [14] and 5/121 (4.1%) fully matched to a reported subtype or isolate (Table 1; see also Supplementary Figure 1).

In 45/121 (37.2%) cases, the obtained sequences were not found in GenBank. The SPF_{10} inner primer sequences covering 22 bp had a maximum of one nucleotide difference compared to a specific reported HPV sequence in 42/45 cases. These specimens were considered unreported 'variants'. The 3/45 remaining specimens yielded SPF_{10} sequences with no close homology to one specific HPV type and no flanking sequences were obtained.

The HPV genotypes recognized in 121 cases by sequence analyses are listed in Table 1. HPV26, 30 and 67 were the most prevalent genotypes among the 121 sequenced cases. The reference HPV67 SPF_{10} sequence was found in seven ICC cases, while the same nucleotide variation was observed in 18 specimens.

In summary, HPV was characterized in 146/198 (73.7%) $LiPA_{25}$-negative cases, either by repeated $LiPA_{25}$ ($n = 25$) or sequence analysis ($n = 121$). The HPV genotype remained unidentified in the other 52 cases, probably due to a low amount of viral target. These cases were named 'HPV undetermined' (HPVU).

Sequencing of ICCs with inconclusive LiP_{25} result

Readable sequences were obtained using primer set II in 102/126 cases (81.0%) with inconclusive $LiPA_{25}$ results and material available. These were used for GenBank comparison by BLAST software; 55 submitted sequences (53.9%) were identical to HPV68 and 43 (42.2%) sequences matched with HPV73 reference type. Four sequences (3.9%) were not reported in GenBank; based on the closest homology, two were assigned as HPV68 and one as HPV18. The remaining sequence could not be reliably assigned to a specific type.

In summary, among 133 cases with inconclusive $LiPA_{25}$ results, sequence analysis demonstrated HPV68 (42.9%), HPV73 (32.3%) and HPV18 (0.8%). The other cases (24.0%) were not tested or negative by sequencing, and the genotyping result remained inconclusive for a specific genotype.

Table 1. Interpretation of HPV sequences from 121 ICC cases

HPV type	Reference type sequence[a] (*n*)	Reported subtype/isolate sequence[b] (*n*)	Unreported sequence (*n*)	Total (*n*)	Total (%)
HPV26	27	0	4	31	25.6
HPV30	26	0	5	31	25.6
HPV67	7	0	19	26	21.5
HPV69	5	0	2	7	5.8
HPV16	0	1	5	6	5.0
HPV82	5	0	1	6	5.0
HPV34	0	2	1	3	2.5
HPV56	0	0	3	3	2.5
HPV68	0	2	0	2	1.7
HPV39	0	0	1	1	0.8
HPV61	1	0	0	1	0.8
HPV91	0	0	1	1	0.8
Unknown HPV	0	0	3	3	2.5
Total (*n*)	71	5	45	121	100

Each sequence was used as a BLAST query for comparison with reported sequences in the GenBank database. Most sequences were identical to an HPV reference genotype or a reported subtype/isolate. Other sequences were unreported but had close homology to a specific reference type. Three sequences did not demonstrate strong similarity to a reported HPV type (unknown HPV). [a]Accession numbers of reference HPV genotypes were: X74472 (HPV26), X74474 (HPV30), D21208 (HPV67), AB027020 (HPV69), K02718 (HPV16), AB027021 (HPV82), X74476 (HPV34), X74483 (HPV56), X67161 (HPV68), M62849 (HPV39), U31793 (HPV61) and AF131950 (HPV91). [b]Accession numbers of reported HPV subtypes/isolates were: AF043287 (HPV16 substrain Phi16), EU918769 (HPV68 isolate LZod68-68) and AJ812226 (HPV34, formerly HPV64 substrain CY24).

Single genotype distribution

Genotyping data obtained on 8977 HPV-positive cases by LiPA$_{25}$ and sequence analysis were combined, as reported previously [1]. Among these cases, 8338 (92.9%) contained a single HPV, while 587 (6.5%) had multiple types, and in 52 cases (0.6%) the HPV remained undetermined. The distributions of single genotypes and α-species among ICC cases are shown in Table 2A, B.

The 13 established carcinogenic types [7] (IARC risk group 1/2A, ie HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) account for 97.54% of the ICC cases with single HPV infections. Phylogenetically related, possibly carcinogenic types (IARC risk group 2B, eg HPV26, 30, 34, 53, 66, 67, 69, 70, 73, 82 and 85) were found (2.25%) through sequencing. All 24 genotypes except HPV85 in the 'carcinogenic' evolutionary group [10] of α-species 5, 6, 7, 9 and 11 were encountered as single infections in ICCs. The remaining genotypes found as single infections in ICC cases belong to a second phylogenetic clade, ie HPV 6, 11, 42, 44, 74 and 91 (0.21%). In the third evolutionary branch, only HPV 61 was found in one case (0.01%).

Mean age at diagnosis and pathology review

The mean age at diagnosis was calculated for the HPV types detected in ICCs (Table 2A). ICC cases with established carcinogenic HPVs (IARC 1/2A) were diagnosed at a mean age of 50.9 years and the possibly carcinogenic types (IARC 2B) at 55.3 years ($p < 0.001$). Table 2B shows that the mean age at diagnosis was significantly lower for α-species 7 (48.5) and 9 (51.4) compared to species 5, 6 and 11 (55.5, 56.8 and 54.4, respectively; $p < 0.05$).

The global mean age at diagnosis was 51 years for all ICC cases in which a single HPV was detected. For the individual HPV types detected in ICC, differences in the mean age at diagnosis were observed. Figure 3 shows the ratio between the mean age at diagnosis per individual type and the global mean age at diagnosis. The 95% confidence intervals are included. ICC cases with single HPV16, 18 or 45 were diagnosed at a relatively younger age (ratio <1), as reported previously [1]. Cases with single HPV69 were also diagnosed at a lower age, whereas types 34 and 53 were associated with a relatively high age at diagnosis (ratio = 1.28). Histological review of available ICC cases with a single possibly carcinogenic type (Group 2B) demonstrated that these were typical squamous cell carcinomas. Many ICCs, particularly those with HPV34 and 26, were keratinizing and relatively well differentiated (Table 3).

Discussion

A newly developed sequence methodology demonstrated excellent performance in recognizing rare HPV types that are usually not targeted by genotyping assays. Among a worldwide collection of 8338 HPV-positive invasive cervical carcinoma (ICC) specimens, 2.25% contained a single HPV genotype classified by IARC as possibly carcinogenic (ie HPV26, 30, 34, 53, 66, 67, 69, 70, 73 and 82). These HPVs were detected in typical squamous cell carcinomas diagnosed at a relatively high mean age.

This paper provides new evidence for the closeness of the link between HPV phylogeny, carcinogenicity and invasive cervical carcinoma [10]. Single presence in ICCs of HPV genotypes classified by the IARC as possibly carcinogenic (Group 2B) provides evidence for a causal association. These types have a close phylogenetic relationship with established carcinogenic genotypes (Group 1/2A), ie HPV16, 18, 31, 33, 35,

Table 2. (A) Overview of anogenital HPV genotypes in relation to the carcinogenic classification by IARC, the single type distribution among ICC, and the mean age at diagnosis for ICC cases with a single genotype ($n = 8338$). (B) The single type distribution among ICC and the mean age at diagnosis, represented according to species and evolutionary clade

(A)

HPV genotype	IARC carcinogenic group	Single type distribution among ICC ($n = 8338$) (n)	(%)	Cases with age information (n)	Mean (median*) age at diagnosis (years)	Minimum–maximum age at diagnosis (years)
HPV6	3	9	0.11	8	47.5*	31–77
HPV11	3	2	0.02	2	40.0*	36–44
HPV13	3					
HPV74	3	1	0.01	1	48*	48
HPV44	3	1	0.01	1	57*	57
HPV55	3					
HPV91	3	1	0.01	0	–	–
HPV7	3					
HPV40	3					
HPV32	3					
HPV42	3	3	0.04	2	75.0*	72–78
HPV54	3					
HPV52	1	226	2.71	192	60.0	25–95
HPV67	2B	26	0.31	24	56.0	35–82
HPV33	1	319	3.83	264	57.1	24–91
HPV58	1	185	2.22	166	58.5	26–86
HPV16	1	5115	61.35	4295	50.1	17–97
HPV31	1	304	3.65	264	54.0	20–92
HPV35	1	162	1.94	121	54.8	30–86
HPV34	2B	6	0.07	6	69.0*	42–87
HPV73	2B	43[a]	0.52	40	52.8	31–82
HPV59	1	90	1.08	87	50.9	26–89
HPV18	1	857	10.28	728	48.3	23–87
HPV45	1	474	5.68	407	47.0	22–84
HPV70	2B	9	0.11	8	52.0*	41–75
HPV39	1	139[b]	1.67	128	51.2	24–81
HPV68	2A	87[a]	1.04	70	51.8	29–90
HPV85	2B					
HPV26	2B	31	0.37	26	55.5	32–78
HPV69	2B	7	0.08	7	50*	31–66
HPV51	1	104	1.25	80	56.0	27–87
HPV82	2B	6	0.07	6	53.5*	36–81
HPV30	2B	31	0.37	23	51.5	26–78
HPV53	2B	22	0.26	13	65.4	36–82
HPV56	1	70	0.84	60	56.8	26–86
HPV66	2B	7	0.08	5	60*	33–74
HPV57	3					
HPV2a	3					
HPV27	3					
HPV71	3					
HPV90	3					
HPV61	3	1	0.01	1	65*	65
HPV72	3					
HPV62	3					
HPV81	3					
HPV83	3					
HPV89	3					
HPV84	3					
HPV86	3					
HPV87	3					
HPV28	3					
HPV3	3					
HPV10	3					
HPV29	3					
HPV77	3					
Total		8338	100	7035	51.0	17–97

*Median age for categories with low number of cases or exact age for categories with only one value. [a]In 28 specimens the $LiPA_{25}$ result was HPV68 or 73, and sequence analysis was unsuccessful. These cases are considered positive for HPV 68 in this table. [b]In three specimens the $LiPA_{25}$ result was HPV39 or 68 or 73, and sequence analysis was unsuccessful. These cases are considered positive for HPV39 in this table.

Table 2. (*Continued*)

(B)

HPV species[a]	Distribution of single types among ICC (n = 8338) Per clade (n)	Per clade (%)	Per species (n)	Per species (%)	Cases with age information (n)	Mean (median*) age at diagnosis (years)	Minimum–maximum age at diagnosis (years)
α-10			13	0.16	12	47.5*	31–77
α-8			1	0.01	0	–	–
α-1	17	0.21	3	0.04	2	75.0*	72–78
α-13							
α-9			6337	76.00	5326	51.4	17–97
α-11			49	0.59	46	54.4	31–87
α-7	8320	99.78	1656	19.86	1428	48.5	22–90
α-5			148	1.78	119	55.5	27–87
α-6			130	1.56	101	56.8	26–86
α-4							
α-15							
α-3	1	0.01	1	0.01	1	65*	65
α-2							

*Median age for categories with low number of cases or exact age for categories with only one value. [a]Comprising: α-10 (HPV6, 11, 13, 74, 44, 55), α-8 (HPV91, 7, 40), α-1 (HPV32, 42), α-13 (HPV54), α-9 (HPV52, 67, 33, 58, 16, 31, 35), α-11 (HPV34, 73), α-7 (HPV59, 18, 45, 70, 39, 68, 85), α-5 (HPV26, 69, 51, 82), α-6 (HPV30, 53, 56, 66), α-4 (HPV57, 2a, 27), α-15 (HPV71, 90), α-3 (HPV61, 72, 62, 81, 83, 89, 84, 86, 87) and α-2 (HPV28, 3, 10, 29, 77). Both parts of the table are adapted from Schiffman *et al* [10] and also contain previous results from de Sanjose *et al* [1].

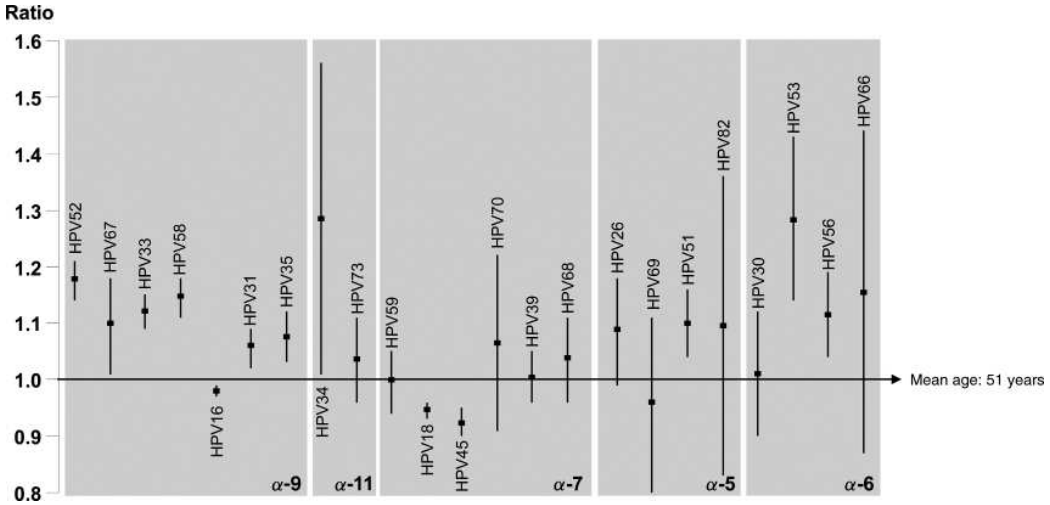

Figure 3. Ratio between mean age at diagnosis per type and the global mean age at diagnosis for ICC cases in which a single HPV from α-species 5, 6, 7, 9 and 11 was detected. The 95% confidence intervals are included. This figure is also based on previous results from de Sanjose *et al* [1].

39, 45, 51, 52, 56, 58, 59 and 68. Carcinogenic HPVs accounted for 97.54% of the single genotypes identified. Together, all types except HPV85 belonging to 'carcinogenic' α-species 5, 6, 7, 9 and 11 [8,10] were encountered as single infections in ICC (99.79%).

Although HPV85 can be amplified by SPF_{10} primers and recognized by conservative DEIA probes (data not shown), it was the only possibly carcinogenic type not detected in this study panel. In a meta-analysis of HPV type-specific prevalence data in ICCs [15], HPV85 had the lowest prevalence of all types from the 'carcinogenic' α-species.

The sequence methodology provided novel sequences that were not identical but had a high degree of homology to a known HPV type. The minor nucleotide variations suggest the presence of a novel HPV variant; this needs to be confirmed by formal phylogenetic classification [16,17], although this is difficult in these old FFPE specimens.

The possibly carcinogenic HPVs were detected in typical squamous cell carcinomas, generally keratinizing and well differentiated. The mean age at diagnosis was higher compared to established carcinogenic genotypes (eg HPV16, 18 and 45). HPVs in α-species 5, 6, 7, 9 and 11 are all associated with ICCs, but the differences in prevalence, mean age at diagnosis and type of ICC are not well understood.

Mechanistic evidence for the carcinogenic potential of all types in the 'carcinogenic' clade is limited. Recent studies have demonstrated that all HPVs within α-species 5, 6, 7, 9 and 11 contain a form of the E6 oncoprotein that can promote the degradation of p53,

Table 3. Pathology review results of ICC cases with a single, possibly carcinogenic, HPV type (IARC risk group 2B)

HPV type	Species	Available cases	Typical SCCs [*n* (%)]	Keratinizing SCCs [*n* (%)]	Papillary and keratinizing SCCs [*n* (%)]	Papillary and non-keratinizing SCCs [*n* (%)]	Warty SCCs [*n* (%)]	ADS[a] [*n* (%)]
HPV67	α-9	25	17 (68)	7 (28)	1 (4)	0	0	0
HPV34	α-11	4	0	4 (100)	0	0	0	0
HPV73	α-11	14	9 (64)	5 (36)	0	0	0	0
HPV70	α-7	9	8 (89)	0	0	0	0	1 (11)
HPV85	α-7	–	–	–	–	–	–	–
HPV26	α-5	25	6 (24)	17 (68)	2 (8)	0	0	0
HPV69	α-5	6	5 (83)	1 (17)	0	0	0	0
HPV82	α-5	6	4 (67)[b]	0	0	2 (33)	0	0
HPV30	α-6	25	9 (36)	14 (56)	2 (8)	0	0	0
HPV53	α-6	19	2 (11)	12 (63)[c]	1 (5)	1 (5)	3 (16)[d]	0
HPV66	α-6	6	4 (67)	2 (33)	0	0	0	0

[a]ADS, adenosquamous carcinoma. [b]Including one case with basaloid features. [c]Including two cases with basaloid features. [d]Including one case with basaloid features.

a tumour suppressor protein, while types from other species do not [18,19]. In addition, the HT-3 cervical carcinoma cell line contains DNA of HPV30, a possibly carcinogenic genotype. Introduction and expression of the bovine papillomavirus E2 protein repressed the endogenous HPV30 *E6/E7* genes and activated a complex growth-inhibitory programme [20]. These results were analogous to those of previous studies on the HeLa cervical cancer cell line, which contains the carcinogenic HPV18 [21–23].

A type belonging to α-species other than 5, 6, 7, 9 and 11 was detected in a very small proportion (0.22%) of ICC cases (ie HPV6, 11, 42, 44, 61, 74 and 91). These types are generally considered non-carcinogenic, as they are found frequently in benign lesions but rarely in malignancies. These types have an association with specific tumour types and their carcinogenic mechanism appears to be different from genotypes in 'carcinogenic' α-species 5, 6, 7, 9 and 11, on the basis of p16INK4a expression and E7 pRB-binding site motif (manuscript submitted for publication).

In addition, the E6 proteins of carcinogenic HPV types have a motif to bind cellular targets containing a PDZ (PSD95/Dlg/Zo-1) domain, which is absent in the E6 proteins of non-carcinogenic HPVs [24,25]. Loss or mislocalization of PDZ proteins correlates with the loss of cell polarity, thus enhancing invasiveness and metastasis [24,26]. Other characteristics that distinguish carcinogenic from non-carcinogenic HPVs are telomerase activation for indefinite life span [27], induction of chromosome instability [28] and viral chromosomal integration [29,30]. However, most of the studies on molecular interactions of E6 and E7 proteins have focused only on HPV16 and 18 and to a limited extend on HPV6 and 11 [26,31,32].

Our study has several limitations. Possibly carcinogenic HPV types were recognized in ICCs by sequence analysis of FFPE tissue specimens with variable quality of DNA. Therefore, only small regions were designed for amplification, and the amount of DNA sequence information available for HPV type recognition was limited. In addition, the detection of a single HPV in ICC provides only circumstantial evidence for a carcinogenic role. Biological activity of HPV, eg transcriptional activity of the viral oncogenes *E6* and *E7*, was not investigated in these archival specimens.

Further research is required to establish the role of possibly carcinogenic HPVs in carcinogenesis. These are too low in prevalence for extensive epidemiological studies. Feasible mechanistic studies, analogous to those performed on HPV16, include p16INK4a immunohistochemistry to determine whether expression patterns are similar to ICCs caused by established carcinogenic types, detection of *E6* or *E7* mRNA, and pRB-binding site affinity and transformation capacity.

Case-control studies could help elucidate risk associations, but are challenging. A recent meta-analysis estimated the worldwide prevalence of HPV types in 215 568 women with normal cytology [33]. Although various genotyping methods were used and not all types were included, the type-specific prevalence of possibly carcinogenic types was very low (varying in the range 0.2–0.6%) in normal women. This is in contrast with non-carcinogenic types such as HPV6 and 11, which are rare in malignancies but frequent in benign lesions.

From the clinical point of view, possibly carcinogenic types individually have a small role compared to the 13 carcinogenic HPV types. The prevalence of several possibly carcinogenic types did not exceed that of HPV6, ie HPV34, 66, 69, 70, 82 and 85. However, the contribution of possibly carcinogenic HPV types as a group (2.25%) is greater than that of HPV35, 39, 51, 56, 58, 59 and 68 individually. Cross-protection by HPV vaccines against types that are closely related to HPV16 and 18 may provide an increased benefit for women. The extent to which the current vaccines will protect against the rare types in α-species 7 and 9 has not been investigated.

It remains questionable whether rare, possibly carcinogenic types should be directly included as targets of HPV tests for cervical cancer screening and triage. HPV vaccines are expected to reduce the incidence of HPV16- and HPV18-related cervical cancers.

If there is no vaccine prevention, the relative importance of the possibly carcinogenic HPVs in causing ICC is likely to increase. Others have evaluated the disadvantageous trade-off in decreased clinical specificity over increased sensitivity when testing for more than about 10 carcinogenic HPV types [34,35]. A better understanding of the relative prevalence of possibly carcinogenic types among women with normal cytology, precancerous lesions and ICCs is needed to clarify decisions about possibly carcinogenic types.

Our findings support the suggested close link between HPV phylogeny and carcinogenicity [8,35]. Possibly carcinogenic types are found as single infections in ICCs, although their relative infrequency makes their clinical importance currently substantially less than established carcinogenic types.

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Author contributions

DG participated in the study design, data collection, data analysis, interpretation of the results and writing of the report; WQ, XB and SS participated in the study design, data collection, interpretation of the results and writing of the report; LA participated in the data collection, data analysis, interpretation of the results and writing of the report; AM participated in the data collection and writing of the report; DJ, NG and MK participated in the data analysis, interpretation of the results and writing of the report. All authors provided approval of the final draft of the report.

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SUPPLEMENTARY MATERIAL ON THE INTERNET

Supplementary Figure 1. Alignment of 121 SPF_{10} sequences versus the reference type with closest homology. If available, flanking sequences were included.