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Diversity of human papillomaviruses in skin lesions

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ABSTRACT

Pools of frozen biopsies from patients with squamous cell carcinoma (SCC) ($n=29$) actinic keratosis (AK) ($n=31$), keratoacanthoma ($n=91$) and swab samples from 84 SCCs and 91 AKs were analysed with an extended HPV general primer PCR and high-throughput sequencing of amplimers. We found 273 different HPV isolates (87 known HPV types, 139 previously known HPV sequences (putative types) and 47 sequences from novel putative HPV types). Among the new sequences, five clustered in genus Betapapillomavirus and 42 in genus Gammapapillomavirus. Resequencing of the three pools between 21 to 70 times resulted in the detection of 283 different known or putative HPV types, with 156 different sequences found in only one of the pools. Type-specific PCRs for 37 putative types from an additional 296 patients found only two of these putative types. In conclusion, skin lesions contain a large diversity of HPV types, but most appeared to be rare infections.

Introduction

More than 150 different human papillomavirus (HPVs) types have been completely cloned, sequenced and given an official number and the number of putative novel HPV types is continuously growing (Bernard et al., 2010; Bottalico et al., 2011; Chen et al., 2007a; Chen et al., 2007b; Chouhy et al., 2010; Ekstrom et al., 2011; Ekstrom et al., 2010; Foulongne et al., 2012; Kohler et al., 2011; Li et al., 2012; Nobre et al., 2009; Vasiljevic et al., 2008; Vasiljevic et al., 2007). The oncogenic mucosal types of HPV cause cervical cancer (Walboomers et al., 1999), as well as vulvar, anal and penile cancer (IARC, 2007) whereas some cutaneous HPV types cause skin warts and others are associated with development of squamous cell carcinoma (SCC) in patients with the rare immunosuppressive disease epidermodysplasia verruciformis (Jablonska et al., 1972, 1997). Several cutaneous HPV types are commonly found in different skin lesions such as squamous cell carcinoma (SCC) (Asgari et al., 2008; Harwood et al., 2004), actinic keratosis (AK) (Mackintosh et al., 2009), and keratoacanthoma (KA) (Forslund et al., 2003; Stockfleth et al., 1999) as well as on healthy skin (Antonsson et al., 2000; de Koning et al., 2007, 2009; Forslund et al., 2004), in both immunocompetent and immunosuppressive patients.

Classification of papillomaviruses (PVs) is based on the sequence of the major capsid protein gene L1, where the sequence of a new HPV type should be $<90\%$ similar to the L1 gene in any known type (de Villiers et al., 2004). Several general primer PCR systems targeting the L1 gene can amplify a broad range of HPV types (Berkhout et al., 1995; de Roda Husman et al., 1995; Forslund et al., 1999, 2003; Gravitt et al., 2000; Harwood et al., 1999). The general primer pair FAP59/64 amplifies both genital and cutaneous types within the HPV genera Alpha-, Beta- and Gammapapillomavirus (Forslund et al., 1999).

Several different methods for type-specific HPV detection exist (Forslund et al., 1994; Poljak et al., 1999; Schmitt et al., 2006; Soderlund-Strand et al., 2008), notably PCR followed by hybridization to type-specific probes coupled to fluorescent beads (Michael et al., 2011; Schmitt et al., 2011), type-specific PCR and general primer PCR, followed by sequencing (Berkhout et al., 1995; Chouhy et al., 2010; Ekstrom et al., 2010; Forslund et al., 2007).

Previously, we found that high throughput sequencing of amplimers obtained using FAP59/64 revealed an extended diversity of HPV types (Ekstrom et al., 2011). In this study, we used bidirectional sequencing using the recently developed 454 Titanium chemistry to investigate if the detection of HPV sequences

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could be improved. In addition, we wished to investigate if the new HPV sequences were commonly present in samples from different skin lesions. The pooled PCR products were purified using MinElute PCR Purification kit (Qiagen), and sequenced using a 454 GS Junior (Roche, Mannheim, Germany).

Results

Identification of known and previously unknown HPV sequences

The analyses of the three different sample pools with different set of primer combinations identified 273 (228 with FAP and 90 with the novel primer combinations) different HPV types or putative types out of which 87 (76 detected with FAP and 33 with novel primer combinations) were known HPV types, 139 (117 detected with FAP and 45 with novel primer combinations) sequences from previously known putative HPV types and 47 (35 with FAP and 12 with novel primer combinations) subgenomic sequences putatively representing novel types (Tables 1 and 2). In a recent report we identified 44 subgenomic sequences from novel putative HPV types, designated SE1 to SE44, of lengths varying from 84 to 450 bp (Ekstrom et al., 2011). In the present study, we used bidirectional sequencing with the GS FLX Titanium chemistry and obtained much longer sequences than in the previous study (mean read length 395 base pairs, compared to 230 base pairs in our previous study). The longer sequences revealed that seven of the previously reported subgenomic SE sequences belonged to the same virus as other SE sequences (SE4=SE27; SE13=SE36; SE17=SE43; SE18=SE29=SE37; SE20=SE44; and SE31=SE33). Sequences SE2, SE6, SE8, and SE21, which were considered as preliminary in the previous publication because of insertion/deletion errors causing premature stop codons in the L1 ORF (Ekstrom et al., 2011), were detected again, but now with more high quality sequences that did not contain premature stop codons. Out of the other 37 SE types detected in the previous study, 33 with FAP and 13 with novel primer combinations were detected again (Table 2). For 17 of these sequences (SE2, 3, 4, 8, 12, 13, 17, 18, 24, 25, 33, 34, 35, 38, 39, 41 and 42), we obtained longer sequences. The longer SE38 sequence was found to overlap with the subgenomic sequence GC04, previously described by Chouhy et al. (2010).

For 33 of the 47 novel putative HPV types detected in this study we obtained >331 bp (range 331–444 bp and mean 436 bp) and for the 14 additional sequences we obtained a sequence from either the 5′-end (n=10, 141–363 bp, and mean=262 bp) or the 3′-end of the amplimer (n=4, 222–225 bp, and mean=224 bp) (Table 1).

The pool with swab samples from SCCs and AKs contained most of the sequences from known/putative HPV types (n=163) closely followed by the biopsies from KAs (known/putative HPV types: n=150), both pools contained similar number of novel putative HPV types (n=24) (Table 1). The majority of the known HPV sequences found belonged to HPV types in genus Gammapapillomavirus (n=128), followed by genus Betapapillomavirus (n=73) and genus Alphapapillomavirus (n=25) (Table 1). Based on the genera of the closest hit in BLAST, most of the novel putative HPV types also belonged to the genus Gammapapillomavirus (n=42), five sequences belonged to genus Betapapillomavirus and none to genus Alphapapillomavirus.

Resequencing

The three sample pools (A, B and C) were resequenced using 121 different multiplex identifiers (MIDs).

An adjusted value for the number of reads from each MID was calculated by multiplying the number of reads for each MID with the number of MIDs used in that particular MID-pool, i.e. 21, 30, 35 and 35. The average adjusted number of reads was 77305 (median 71,820 and range 21,595–280,000). One MID that generated only 3605 reads was considered inadequate and was excluded.

Altogether sequences from 283 different known and putative HPV types were detected. Sample A (fresh frozen biopsies from 29 SCCs and 31 AKs) was resequenced using 30 different MIDs. Sequences from 93 known and putative HPV types were detected, but only three types were detected by all the 30 MIDs (Table 3). Twenty types/putative types were detected by more than 50% of the analyses. Sample B (fresh frozen biopsies from 91 KAs) was resequenced using 70 different MIDs in two separate sequencing runs. In total, 198 known and putative HPV types were detected (Table 3). Sequences from 59 HPV types/putative types were detected by more than half of the resequencing, but only six types were detected in all 70 MIDs. Pool C (swab samples from the top of 84 SCCs and 91 AKs) was resequenced with 21 different MIDs. In

Table 1
Number and genera of HPV sequences found.

Sample	Primers	Known HPV sequences				Novel HPV sequences[a]			Genera of known HPV sequences[d]			Genera of novel putative HPV types[d]	
		HPV types	FA types	FAIMVS	SE types	Complete fragment[a]	Partial, 5′	Partial, 3′	α	β	γ	β	γ
Frozen biopsies from 29 SCCs[b] and 31 AKs[b]	FAP59/FAP64	36	31	3	10	4	1	0	1	47	32	1	4
	Novel primers	13	11	0	0	1	0	0	0	13	11	0	1
Frozen biopsies from 91 KAs[b]	FAP59/FAP64	63	50	7	21	13	6	3	23	43	75	2	20
	Novel primers	12	13	0	3	2	0	0	1	13	14	0	2
Swab samples from 84 SCCs and 91 AKs	FAP59/FAP64	46	62	6	24	9	5	1	2	55	80	1	14
	Novel primers	26	17	2	10	9	0	0	0	35	20	2	7
All samples[c]		87	91	8	40	33	10	4	25	73	128	5	42

[a] >400 bp sequences represent an almost complete or a complete FAP amplimer. Partial sequences may map to either the 5′ or the 3′ of the FAP amplimer.
[b] SCC=squamous cell carcinoma, AK=actinic keratosis, and KA=keratoachantoma.
[c] As sequences were found in more than one of the pools the sum of the sequences is less than the total number of sequences.
[d] Genera based on the top hit sequence in BLAST.