Detection of human papillomavirus (HPV) L1 gene DNA possibly bound to particulate aluminum adjuvant in the HPV vaccine Gardasil®

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ABSTRACT

Medical practitioners in nine countries submitted samples of Gardasil® (Merck & Co.) to be tested for the presence of human papillomavirus (HPV) DNA because they suspected that residual recombinant HPV DNA left in the vaccine might have been a contributing factor leading to some of the unexplained post-vaccination side effects. A total of 16 packages of Gardasil® were received from Australia, Bulgaria, France, India, New Zealand, Poland, Russia, Spain and the United States. A nested polymerase chain reaction (PCR) method using the MY09/MY11 degenerate primers for initial amplification and the GP5/GP6-based nested PCR primers for the second amplification were used to prepare the template for direct automated cycle DNA sequencing of a hypervariable segment of the HPV L1 gene which is used for manufacturing of the HPV L1 capsid protein by a DNA recombinant technology in vaccine production. Detection of HPV DNA and HPV genotyping of all positive samples were finally validated by BLAST (Basic Local Alignment Search Tool) analysis of a 45–60 bases sequence of the computer-generated electropherogram. The results showed that all 16 Gardasil® samples, each with a different lot number, contained fragments of HPV-11 DNA, or HPV-18 DNA, or a DNA fragment mixture from both genotypes. The detected HPV DNA was found to be firmly bound to the insoluble, proteinase-resistant fraction, presumably of amorphous aluminum hydroxyphosphate sulfate (AAHS) nanoparticles used as adjuvant. The clinical significance of these residual HPV DNA fragments bound to a particulate mineral-based adjuvant is uncertain after intramuscular injection, and requires further investigation for vaccination safety.

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1. Introduction

The quadrivalent human papillomavirus (HPV) vaccine, Gardasil® (Merck & Co.), has been recommended for prevention of HPV-initiated cervical cancer and precancers since 2006 [1]. The active ingredients in the vaccine are genotype-specific HPV L1 capsid proteins in the form of virus-like-particles (VLPs), which are highly effective in eliciting antibody production in the host against future infection by HPV-16, HPV-18, HPV-6 and HPV-11 and contain no viral DNA [2,3]. These VLPs are produced by a DNA recombinant technology in which the genotype-specific "viral genes coding for the capsid proteins" [4] are inserted into the plasmid pGAL110 for transformation of the yeast spheroplasts [5]. For the vaccine to be effective, young girls age 9–12 are targeted for vaccination before their sexual activity begins [6].

According to the records kept by the U.S. Centers for Disease Control and Prevention (CDC), an apparently high number of side effects have been reported following HPV vaccinations in certain categories of health disorders [7]. Using the Brighton case definition of anaphylaxis for diagnostic certainty, the estimated rate of anaphylaxis in young women after HPV vaccination was found to be 5 to 20 times higher than those identified in comparable school-based vaccination programs

[8]. Rheumatoid arthritis, including juvenile rheumatoid arthritis, was recorded 3 times more frequently in the Gardasil®-vaccinated subjects than in the control group receiving amorphous aluminum hydroxyphosphate sulfate (AAHS) adjuvant during clinical trials [9]. A number of cases of possibly immune-based inflammatory neurodegenerative disorders involving the central nervous system, known as acute disseminated encephalomyelitis, following Gardasil® injections have been reported in world literature [10–16]. Physicians from several countries submitted samples of this quadrivalent HPV vaccine-currently being used in the market- to the author's laboratory contracted by a nonprofit organization (SANE VAX Inc.) to be tested for the presence of HPV DNA in the vaccine samples. These health care providers and some of their patients suspected that residual recombinant HPV DNA left in Gardasil® might have contributed to some of the unexplained post-vaccination side effects.

To clarify the vaccine specification, the U.S. Food and Drug Administration has recently announced that Gardasil® indeed does contain recombinant HPV L1-specific DNA fragments [17]. However, the physical conditions of these HPV DNA fragments in the final vaccine products have not been characterized. It is not clear if they are in the form of free HPV DNA molecules in the aqueous phase of a suspension, encapsulated inside the VLPs [18,19], reversibly bound to the insoluble AAHS adjuvant as the VLPs, or irreversibly bound to the mineral aluminum (Al^{3+}) [20]. Free foreign DNA molecules are

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known to be degraded and eliminated quickly by the mammalian hosts [21]. The DNA fragments encapsulated in the VLPs [22,23] or bound to the particulate aluminum adjuvant [24] may be delivered into the antigen-presenting cells or macrophages after injection. Their physical condition in the vaccine may determine the fate of these foreign DNA fragments in a vaccinated person and their variable physiopathological effects on the host. Different inorganic aluminum compounds with their specific physicochemical characteristics have been a subject of intense research [24–27] because they can boost the host's immunity response to both protein-based [27] and DNA-based [24,28] vaccination.

This article reports the results of HPV DNA testing performed on 16 samples of Gardasil® – each bearing a different batch lot number received from 9 countries – and shows that the residual HPV L1 gene DNA fragments are probably firmly bound to AAHS nanoparticles.

2. Experimental

Since Gardasil® is a prescription drug, all samples tested were purchased by licensed medical practitioners in the country of origin from their respective local drug suppliers. Gardasil® is marketed in 0.5-mL suspensions for injection in a single-dose vial and in a manufacturer-sealed prefilled syringe. A total of 16 Gardasil® samples with intact original packages, including 3 unopened vials and 13 unopened prefilled syringes were received from physicians in Australia, Bulgaria, France, India, New Zealand, Poland, Russia, Spain and the United States, each bearing one of the following lot numbers: #1437Z, #1511Z, # 0553AA, #NL35360, #NP23400, #NN33070, #NL01490, #NM25110, #NL39620, #NK16180, #NK00140, #NM08120, #NL13560, #NL49190, #NN28160, or #NM29390. The 3 samples sent from the medical doctors located in the U.S. were delivered to the laboratory with cold-packs in thermally insulated containers. The other 13 samples from various countries outside the U.S. were transported in non-insulated containers exposed to ambient temperatures.

A commonly targeted region of the HPV L1 gene DNA was first amplified by a primary PCR using the MY09/MY11 degenerate primer pair, followed by a nested PCR using a pair of GP5/GP6, a pair of GP6/MY11, or a pair of GP5/MY09 general consensus primers. Three primer pairs were chosen to perform the second PCR to amplify multiple nests of the 450 bp MY09/MY11 PCR products in an attempt to cover possible sequence variants of the genotype-specific L1 genes that are used for manufacturing of the quadrivalent HPV vaccines by the DNA recombinant technology [29], but may not be identical to those which the GP5/GP6 general primers were designed for. The PCR products were visually identified by standard agarose gel electrophoresis. The relative positions of these primer-binding sites in the open reading frame of the HPV L1 gene, the sequences of the primers used, and the expected

size of the amplicon terminated by each primer pair are summarized in Fig. 1. The presumptive nested PCR amplicons of HPV DNA were subjected to automated direct DNA sequencing, using a GP6 or a GP5 oligonucleotide as the sequencing primer. A segment of 45–60 bases of the hypervariable region of the L1 gene sequence was excised from the computer-generated base-calling electropherogram for BLAST analyses for confirmation of the HPV DNA detected and to validate its genotype. The technical detail of this nested PCR/DNA sequencing methodology has been previously reported [30–35].

Experiments were first designed to determine if free HPV DNA was detectable in the solution of the Gardasil® vaccine. To this end, an aliquot of 100 μL of the vaccine suspension was centrifuged at ~16,000×g for 10 min in a 1.5 mL microcentrifuge tube at room temperature. The entire supernatant was transferred to another 1.5 mL microcentrifuge tube containing 500 μL of 95% ethanol, 12 μL of water, and 68 μL of 3 M sodium acetate. After the pellet was washed 3 times with 1 mL of 70% ethanol each and the final ethanol suspension was centrifuged at ~16,000×g for 5 min, the pellet was air-dried. The dried pellet was re-suspended in100 μL of 0.1 mg/mL proteinase K (Sigma Chemical Co., St. Louis, MO) in a buffer consisting of 50 mM Tris–HCl, 1 mM EDTA, 0.5% Tween 20, pH 8.1. The mixture was digested at 45–55 °C overnight. After inactivation of the proteinase K solution in a metal block heated at 95 °C for 10 min, 1 μL of the unspun digestate was used for each primary PCR followed by nested (or hemi-nested) PCR with various nested PCR primer pairs described above.

To test for HPV DNA in the insoluble part of the Gardasil® vaccine, the pellet of the centrifuged 100 μL vaccine suspension described above was washed twice with 1 mL of 70% ethanol each and the final ethanol suspension was centrifuged at ~16,000×g for 5 min. The washed pellet was air-dried. The dried pellet was re-suspended in100 μL of proteinase K solution. The suspension was digested at 45–55 °C overnight and centrifuged at ~16,000×g for 5 min the next day. The supernatant of the digestate was transferred to a 1.5 mL centrifuge tube and was heated at 95 °C for 10 min to inactivate the proteinase K. One microliter of the unspun digestate supernatant was used for each primary PCR followed by nested PCR.

The pellet of the proteinase K digestate of the insoluble part of Gardasil® was exhaustively washed with the buffer solution (50 mM Tris–HCl, 1 mM EDTA, 0.5% Tween 20, pH 8.1) 4 times, 1 mL each time. The final washed pellet, presumably consisting of protein-free AAHS particles, was re-suspended in 100 μL of buffer. After heating to 95 °C for 10 min, 1 μL of the washed and heated insoluble particle suspension was used for each primary PCR followed by nested PCR.

Short target sequence HPV genotyping was performed by direct automated cycle DNA sequencing [30–35]. Briefly, a trace of the positive nested PCR product was transferred directly with a micro-glass rod

Hypervariable region of the HPV L1 gene terminated by MY09 and MY11 primers

………//………..MY09………………GP6…………...GP5…MY11………..//…

(Size of PCR amplicon between MY09 and MY11 is ~450 bp, not drawn to scale)

MY09, MY11, GP5 and GP6 are annealing sites for PCR primers with their base sequences listed below:

MY09 = 5'-CGTCCMARRGGAWACTGATC-3'
MY11 = 5'-GCMCAGGGWCATAAYAATGG-3'
Key to degenerate nucleotides: M=(A+C), R=(A+G), W=(A+T), Y=(C+T)

GP5 = 5'-TTTGTTACTGTGGTAGATAC-3'

GP6 = 5'-GAAAAATAAACTGTAAATCA-3'

Number of nucleotides from GP5 to GP6 ~150 bases (genotype-dependent)
Number of nucleotides from GP6 to MY11=181-190 bases (genotype-dependent)
Number of nucleotides from GP5 to MY11=65 bases (INNO-LiPA PCR amplicon) [52-55]

Fig. 1. Title: position map of four PCR primers for the detection of HPV L1 gene DNA. Description: relative locations of MY09, GP6, GP5 and MY11 primer sites.

from the positive nested PCR tube into a 20 μL volume of a cycle sequencing reaction mixture consisting of 14.5 μL water, 3.5 μL of 5 X buffer, 1 μL of BigDye Terminator 1.1 (Applied Biosystems) and 1 μL of 10 μM GP6, or GP5 sequencing primer. After thermal cycling according to the manufacturer's recommendation, the reaction mixture was loaded in an automated ABI 3130 four-capillary Genetic Analyzer for sequence analysis. Alignment analysis of a 45–60 bases sequence in the hypervariable region of the L1 gene excised from the computer-generated base-calling electropherogram was performed against various standard HPV genotype sequences stored in the GenBank, using the on-line BLAST system to validate the specific HPV genotyping.

Extraordinary precautionary steps were taken to ensure that the detection of any HPV target DNA was not due to inadvertent amplification of ambient HPV DNA sequences. A molecular laboratory was dedicated exclusively to this vaccine testing project from June 1 to October 31, 2011. During this period the entire project was completed and no other nucleic acid work was performed in the same facility. Transferring of primary PCR products to the nested PCR mixture and nested PCR products to the Sanger reaction mixture was accomplished with micro-glass rods to avoid micropipetting aerosol and all procedures were carried out by highly trained, experienced molecular technologists according to a set of guidelines for the nested PCR technology applied in clinical diagnostic laboratories [35]. Negative water and primer controls were included in each PCR run of no more than 4 samples in one run. All PCR primers, including the MY09, MY11, GP5 and GP6 oligonucleotides, were tested, as previously described [30], against standard plasmid DNA of HPV type-16, -18, -11 or -6B purchased from American Type Culture Collection to ensure that 1–10 copies of plasmid DNA from each genotype could be detected by the nested PCR protocol designed for this project.

3. Results

Agarose gel electrophoresis of the GP6/MY11 or GP5/GP6 nested PCR products of all 16 Gardasil® samples tested revealed bands of expected size for HPV DNA when the proteinase K-resistant insoluble part of the vaccine, presumably containing HPV DNA fragments bound to AAHS, was used as the template to start the primary PCR. However, primary PCR with the degenerate MY09/MY11 primer pair did not generate a visible PCR product band on any of the samples tested. When the GP5/MY09 primer pair instead of the GP6/MY11 primers was used, no nested PCR products were observed under identical experimental conditions, even when the entire pellet was used for starting a primary PCR. Since there were no primary PCR products observed, it remains questionable if the accumulation of the target DNA copies was numerically exponential or linear in the MY09/MY11 primary PCR.

No PCR products, primary or nested, were obtained when the supernatant of the Gardasil® vaccine or the supernatant of the proteinase K digestate of the insoluble particles of the Gardasil® vaccine was used as the starting material to initiate the primary PCR.

After the above results were obtained, a vial of recombinant hepatitis B vaccine, Recombivax HB® which also uses AAHS as the adjuvant for its formulation [36], was tested in parallel with 4 Gardasil® lot samples to determine if the HPV DNA fragments detected in the Gardasil® vaccine lots might have been a contaminant bound to the AAHS adjuvant ingredients in general use for other vaccine formulations by the manufacturer (Merck & Co.). The results of this parallel comparative nested PCR experiment showed no evidence of HPV DNA in the AAHS particles in a vial of Recombivax HB® purchased on the U.S. market (Fig. 2). The latter finding supported the interpretation that the HPV DNA fragments bound to the AAHS particles were associated with the Gardasil® manufacturing process, not a contaminant of the adjuvant used in the vaccine formulation.

To determine if the HPV L1 gene DNA detected in the AAHS particulate fraction might be in free solution after re-suspension of the

insoluble particles for PCR amplifications, serial double dilutions of the 4 proteinase K-digested Gardasil® particle suspensions used for the above experiment (Fig. 2) were made in buffer, and 1 μL from each dilution was used as the starting material for primary and then nested PCR. The results showed that the distribution of the HPV L1 gene DNA fragments in these samples was not homogeneous. The concentration of the HPV DNA templates amplifiable by the nested PCR from the dilution ladder did not decrease accordingly while the dilution factors increased progressively toward the endpoint (Fig. 3), as would be expected if free HPV DNA in true solution was titrated by serial dilutions [30]. This finding supported the interpretation that the HPV L1 gene DNA fragments existed in aggregation, which would prevent the success of using serial dilution methods to obtain single HPV DNA molecule samples to perform nested PCR for the preparation of sequencing template on some Gardasil® lots containing HPV DNA molecules of more than one genotype (Fig. 6).

All positive nested PCR amplicons were proven to contain a hypervariable sequence of the L1 gene open reading frame of an HPV-11 DNA synthetic construct (Fig. 4), an African variant of HPV-18 DNA (Fig. 5), or a mixture of these two (Fig. 6). No HPV-6 or HPV-16 DNA residues were detected in this study.

The HPV genotypes detected in the 16 lot samples of Gardasil® are summarized in Table 1. Part of the results presented in this article was previously reported to the FDA by SANE VAX, Inc.

Fig. 2. Title: nested PCR with AAHS recovered from HPV vaccine and hepatitis B vaccine. Description: HPV L1 gene DNA fragments detected in AAHS particles of Gardasil®, but not in AAHS particles of Recombivax HB®. Gel electrophoresis of GP6/MY11 nested PCR products on 4 of the 16 Gardasil® vaccine samples and 1 Recombivax HB® sample (Lot # 0908AA). The MY09/MY11 primary PCR was initiated with 1 μL suspension of the insoluble and proteinase K-resistant fraction derived from each vaccine sample. Visualization of a ~190 bp nested PCR amplicon in lanes #1–4, but not in lane #5, indicates the presence of HPV L1 gene DNA residues possibly bound to the AAHS adjuvant in the HPV vaccine, but not in the AAHS adjuvant of the hepatitis B vaccine. All ~190 bp nested PCR amplicons were confirmed by DNA sequencing to be HPV-11 or HPV-18 DNA or a mixture of both. M = molecular ruler, 100–1000 bp. Lanes 1–4 = Gardasil® GP6/MY11 nested PCR products. Lane 5 = no GP6/MY11 nested PCR products in Recombivax HB®. N = negative water control. P = HPV-16 DNA positive control.
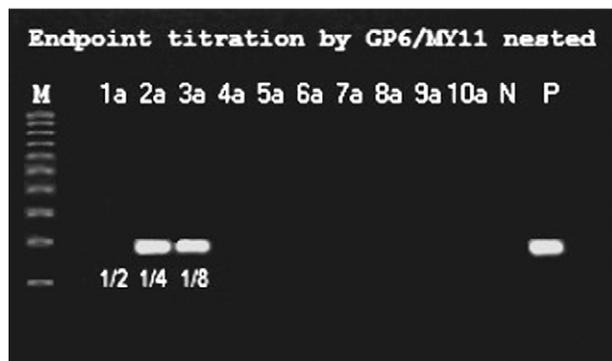
Fig. 3. Title: HPV DNA nested PCR on serially diluted Gardasil® AAHS suspensions. Description: serial dilution experiment suggests HPV DNA bound to insoluble AAHS aggregates. Gel electrophoresis of the nested PCR products after 10 serial double dilutions of 1 of the 4 Gardasil® particulate suspensions (Fig. 2) were used to initiate the respective primary PCRs. Dilution factors covered from ½ in Lane 1a to 1/1024 in Lane 10a. The results showed no PCR product band with sample of a low dilution factor (Lane 1a) followed by heavy product bands of PCR started with samples of higher dilution factors (Lanes 2a and 3a). No gradual reduction in PCR product band density in the dilution ladder was observed, suggesting that the HPV DNA was bound to AAHS particles in solid aggregates. M = molecular ruler, 100–1000 bp. Lanes 1a–10a = GP6/MY11 nested PCR products after the AAHS particle suspension was serially double-diluted from ½ to 1/1024 in buffer. N = negative water control. P = HPV-16 DNA positive control.

4. Discussion

Since the quadrivalent HPV vaccine, Gardasil®, is produced by a DNA recombinant technology in which viral genes coding for the major L1 capsid proteins (4) are inserted into plasmid pGAL110 for transformation of the yeast spheroplasts [5] to manufacture the desired genotype-specific VLPs, any residual HPV DNA detected in the vaccine represents a fragment or fragments of the genetically modified viral DNA for vaccine manufacturing, and not a viable or self-replicating virus.

The presence of HPV DNA in Gardasil® is a surprise to most medical practitioners because this protein-based vaccine has been purified to remove all contaminating components, including viral and plasmid DNA, by a highly effective patented process [37]. According to the published specification of Gardasil®, the active ingredients of the vaccine are "highly purified virus-like particles (VLPs) of the recombinant major capsid (L1) protein of HPV types 6, 11, 16, and 18. As the VLPs do not contain viral DNA, they cannot infect the cells or reproduce [2,3]."

This study shows that the HPV L1 gene DNA fragments are bound to the AAHS nanoparticles, not in the aqueous phase or within the VLPs in the Gardasil® vaccine because they are not detectable in the supernatant of the vaccine, or in the supernatant of the proteinase K digestate of the vaccine precipitates. According to the Gardasil® formulation, the only water-insoluble, proteinase K-resistant excipient in the vaccine is the AAHS precipitates which the manufacturer chooses and specifically prepares as a mineral-based adjuvant. It is inconceivable that an unidentified proteinase-resistant particulate foreign substance could have contaminated all of the Gardasil® vaccine lots tested. Interpretation of the study findings is further explored as follows.

Based on information available in the public domain, in addition to the HPV 6, 11, 16, and 18L1 protein VLPs, Gardasil® contains AAHS adjuvant, sodium chloride, L-histidine, polysorbate 80 (PS-80), sodium borate, and water for injection as excipients [2,3] with a buffer system which provides for a range from pH 6.0 to 6.5 [38]. Except for the VLPs and AAHS adjuvant, all other excipients listed in the formulation are common laboratory chemicals well known for their highly water-soluble properties [39]. The insoluble AAHS adjuvant and VLPs are expected to be in the pellet of the vaccine after centrifugation while all other excipients remain in the supernatant.

If the residual HPV L1 gene DNA fragments existed freely in the aqueous solution as the water-soluble excipients do in the vaccine, they would be rapidly degraded by the nucleases in the serum after intramuscular injection and eliminated from the body of the host (21). However, intramuscular injection of 100 μg of free HPV-16L1 plasmid DNA in BALB/C mice without adjuvant invariably induces a strong CD8 T cell response [27], indicating that under certain conditions free non-replicating HPV L1 gene DNA can activate the immune system.
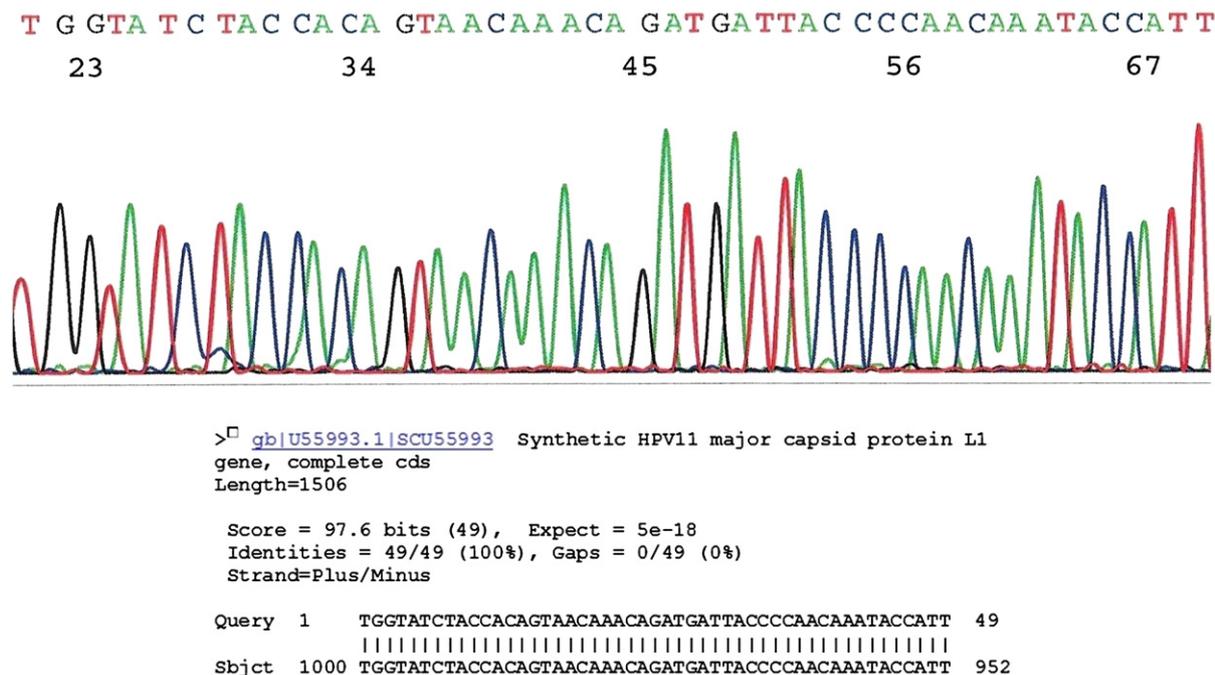
Fig. 4. Title: HPV-11 L1 gene DNA short target sequence. Description: synthetic construct of HPV-11 DNA detected in Gardasil® AAHS adjuvant. HPV-11 L1 gene DNA detected in the insoluble part of Gardasil® after proteinase K digestion and exhaustive washings in detergent buffer pH 8.1. This is the electropherogram of a short target DNA sequencing, using a 181 bp GP6/MY11 nested PCR amplicon as the template. The sequencing primer was GP6. BLAST alignment of 49 bases of the DNA sequence confirmed that the HPV DNA detected was part of the synthetic construct (GenBank Locus SCU55993) designed for production of HPV-11 VLPs used in Gardasil®.

A T T G GTA C TAC G A GT GGTA TCTAC CACA GTAACAA ATAATT GATTAT G CCA GCA GATACCAT T

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>⬚ gb|EF202155.1|  Human papillomavirus type 18 isolate Qv04924, complete
genome Length=7824

 Score = 125 bits (63),  Expect = 3e-26
 Identities = 63/63 (100%), Gaps = 0/63 (0%)
 Strand=Plus/Minus

Query  1     ATTGGTACTACGAGTGGTATCTACCACAGTAACAAATAATTGATTATGCCAGCAGATACC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6609  ATTGGTACTACGAGTGGTATCTACCACAGTAACAAATAATTGATTATGCCAGCAGATACC  6550

Query  61    ATT  63
             |||
Sbjct  6549  ATT  6547
```

Fig. 5. Title: HPV-18 DNA short target sequence. Description: HPV-18 DNA fragment detected in Gardasil® AAHS adjuvant. HPV-18L1 gene DNA detected in the insoluble part of Gardasil® after proteinase K digestion and exhaustive washings. This is the electropherogram of a short target DNA sequencing, using a 187 bp GP6/MY11 nested PCR amplicon as the template. The sequencing primer was GP6. BLAST alignment of 63 bases of the DNA sequence confirmed that the HPV DNA detected was a hypervariable segment of the HPV-18L1 gene (GenBank Locus EF202155).

HPV VLPs are irregularly shaped 30–50 nm structures composed of self-assembled HPV major capsid L1 protein pentamers [40]. VLPs produced by various viral genes, including the HPV VLPs, have been shown to be able to encapsulate non-viral DNA for transfer of the latter into target cells [19,23,41]. If the L1 gene DNA fragments in Gardasil® were encapsulated inside the VLPs, the HPV DNA fragments could be delivered into the antigen-presenting cells (APCs) or macrophages, which may trigger a series of immunological reactions. The VLPs carrying encapsulated DNA can be digested by proteinase K in vitro and release the packaged DNA inside into solution [42].

AAHS is Merck's proprietary mineral-based adjuvant which consists of amorphous precipitates prepared by mixing solutions of NaH_2PO_4, $KAl(SO_4)_2$ and ammonium hydroxide under special controlled conditions (25–27). The mechanism for its extraordinarily high VLP-binding capacity has been investigated (27), but is still not fully understood. It has been attributed to a nonspecific binding due to the unique amorphous mesh ultrastructure of the AAHS precipitates and an electrostatic attraction between the AAHS nanoparticles and the VLPs (27). However, the isoelectric points of the HPV L1 capsid proteins are pH 7.95, 8.35 and 8.55 for HPV-16, HPV-18 and HPV-6, respectively [43], and are positively charged in the Gardasil® vaccine at pH 6.0–6.5. The point of zero charge (PZC) for AAHS is 7, compared to a PZC of aluminum phosphate at pH 5 and a PZC of aluminum hydroxide at pH 10. Yet, the HPV VLP-binding capacity for AAHS is twice as high as that for aluminum phosphate or for aluminum hydroxide (27), indicating that electrostatic attraction plays little role, if any, in the binding between HPV VLPS and AAHS.

The mechanism of binding between AAHS and DNA fragments in Gardasil® is different from that between AAHS and VLPs. DNA molecules are very small, but linear and long, have an isoelectric point at about pH 5.0 and carry a negative charge at pH 6.0–6.5. As a result, DNA molecules in the Gardasil® vaccine can bind to the positively charged AAHS particles electrostatically. However, the HPV L1 gene DNA fragments in the vaccine are detected in the proteinase K-digested precipitates, presumably AAHS nanoparticles which have been exhaustively washed in a nonionic detergent buffer, pH 8.1.

TACGTA SGTA KY G RYA W C WRY A CA R WMA S ATA T TTA T SATTA YR MM T R CC ATT R CCAT T
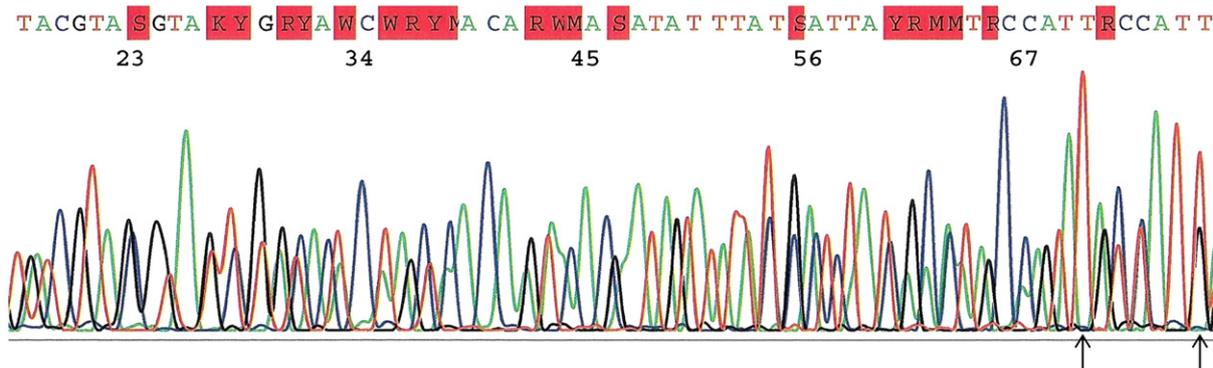
Fig. 6. Title: mixed HPV-11 and HPV-18 short target sequences. Description: mixed HPV-11 and HPV-18L1 gene DNA sequences detected in the insoluble part of a Gardasil® sample after proteinase K digestion and exhaustive washings. This is the electropherogram of a short target DNA sequencing of one of the GP6/MY11 nested PCR products, illustrating two superimposed DNA sequences. Since the GP6/MY11 PCR amplicon for HPV-11 is 181 bp and the GP6/MY11 PCR amplicon for HPV-18 is 187 bp in size [35], some conserved sequences shared by these two genotypes in this composite electropherogram may be recognized in two different positions 6-nucleotides apart. For easy identification, the first 5′ T (counting from the right of the color tracing) in the characteristic CCATT–-5′ ending of the MY11 primer site for these two sequences is pointed with a black arrow. These two Ts are separated exactly six bases apart. Since numerous HPV DNA fragments of both genotypes were firmly bound to any individual AAHS particles, it was not possible to obtain a single DNA fragment by making serial dilutions of the AAHS particles for PCR amplification to prepare a pure DNA template for Sanger sequencing reaction.

Table 1
Gardasil® lot numbers, countries of origin and HPV L1 gene DNA found.

Lot #	Country/source	Genotype
1437Z vial	USA, Connecticut	HPV-11
		HPV-18
1511Z prefilled syringe	USA, New York	HPV-18
0553AA vial	USA, New Jersey	HPV-11
		HPV-18
NL35360 prefilled syringe	France	HPV-11
		HPV-18
NP23400 prefilled syringe	Spain, Valencia	HPV-11
		HPV-18
NN33070 prefilled syringe	Spain, Valencia	HPV-11
		HPV-18
NM25110 prefilled syringe	Australia, Sydney	HPV-11
		HPV-18
NL01490 prefilled syringe	New Zealand, Tauranga	HPV-18
NK16180 prefilled syringe	New Zealand, Northland	HPV-18
NK00140 prefilled syringe	New Zealand, Tauranga	HPV-11
		HPV-18
NM08120 prefilled syringe	New Zealand, Christchurch	HPV-11
		HPV-18
NL13560 prefilled syringe	New Zealand, Wellington	HPV-11
		HPV-18
NL39620 prefilled syringe	Poland	HPV-11
NN28160 vial	Russia	HPV-11
		HPV-18
NL49190 prefilled syringe	Bulgaria	HPV-11
		HPV-18
NM29390 prefilled syringe	India	HPV-18

The latter pH is way above the PZC of the AAHS nanoparticles and the isoelectric point of DNA. Any DNA molecules initially bound to the insoluble AAHS electrostatically would have been washed off because both the DNA molecules and the AAHS particles carry a negative charge at pH 8.1, thus electrostatically repulsive.

The more likely binding mechanism between the HPV L1 gene DNA and the AAHS nanoparticles in Gardasil® is of a chemical nature through ligand exchange of phosphate for hydroxyl, independent of the electrostatic forces [20,44]. When aluminum (Al^{3+}) and DNA interact, the binding site for Al^{3+} on the DNA chains is the phosphate groups on the DNA backbones, not the bases of the DNA molecule [20]. Aluminum-DNA complexes differ from other metal-DNA complexes. Apparently more than one form of DNA can exist at any time in the presence of aluminum. DNA-aluminum complexes formed at different pH are known to have variable DNA melting profiles that only a portion of the DNA is "stabilized" or "denatured" [45], which may explain the success of amplification of the HPV-11 and HPV-18L1 gene DNA fragments when the MY11, GP6 and GP5 PCR primers are used and the failure of amplification with an MY09 PCR primer in the present study. Existent enzyme-based biochemical methods cannot quantify the DNA bound to a particulate aluminum adjuvant. Quantitation of HPV by qPCR assay involves phenol/chloroform extraction of the HPV DNA from proteinase K digestate [46].Such assays cannot be used to quantify the mineral-bound DNA which requires a nested PCR with a highly processive DNA polymerase for detection.

This report provides the first evidence that a chemical binding may have occurred between naked DNA fragments and an aluminum-based adjuvant to form a highly stable complex in a vaccine. However, the evidence is still indirect. Such a new chemical complex may need direct physical analyses of the molecular structure for final validation. A stable HPV DNA–AAHS complex, chemical in nature and protected in the cytoplasm of the macrophages, may explain why HPV L1 gene DNA fragments can be detected in the post-mortem blood and spleen tissue obtained at autopsy after a teenage girl suffered a sudden unexpected death 6 months after receiving the last dose of Gardasil® vaccination (S.H. Lee, manuscript in preparation based on a report submitted to the Coroner of Wellington, New

Zealand for an inquest on August 8–9, 2012). Macrophages laden with particulate aluminum-based adjuvant are known to travel from the site of intramuscular vaccine injection through the blood to the spleen and to other organs [47–49].

One may speculate on the possible answers to the question as to why only residual fragments of HPV-11 and HPV-18 DNA, but not those of HPV-6 or HPV-16 DNA, were detected in a quadrivalent HPV vaccine in the current study. First, it is entirely possible that the nested PCR method using the consensus general primers is not suitable for detecting a small amount of residual HPV-6 and HPV-16 DNA residues bound to the AAHS particles when the HPV-11 and HPV-18 DNA residues are present in overwhelming proportions. Another possibility is that the vaccine manufacturer may have a more effective procedure in cleaning up the HPV-6 and HPV-16 DNA residues from the vaccine products than the procedure used to remove the residues of HPV-11 and HPV-18 DNA fragments. (Author's note — after submission of this manuscript HPV-16L1 gene DNA fragments have been detected in at least some of the Gardasil® vaccine lots using various sets of genotype-specific PCR primers).

All existent commercial test kits and published protocols for HPV DNA assays are designed to test for an analyte in solution. Diligent search of the literature has failed to find a publication dealing with detection of HPV DNA bound to aluminum-based particles. Both the developer [50,51] and the manufacturer [52,53] of the Gardasil® vaccine seem to heavily rely on using the INNO-LiPA kit [54,55] for HPV DNA testing. If the latter procedure was used as the tool to test for HPV DNA residues during vaccine manufacturing, the unrecognized HPV-11 and HPV-18 DNA residues might have been a result of technical failure in detection. In a recently published WHO-sponsored survey, 9 of 12 laboratories using the INNO-LiPA kit were found to be not proficient of detecting the HPV types tested for [56].

As shown in Table 2, the INNO-LiPA probe for detection of the HPV-11 DNA sequence immediately downstream of the MY11 primer region is designed for naturally occurring HPV-11 isolates. In contrast to all known HPV-11 variants isolated from patients, the genetically engineered HPV-11L1 protein-coding synthetic construct for Gardasil® vaccine production has 4 base mismatches against the sequence of the probe in this 22-base interprimer region targeted for hybridization which may fail to take place because the probe is not matched with the target sequence. To allow detection of at least 43 different HPV genotypes in a 22-base sequence region, mismatched hybridization probes must be washed off from the target sequence [54].

Table 2
Mismatched HPV-11 and HPV-18 DNA sequences between the target regions of the HPV DNA in Gardasil® and the INNO-LiPA probes.

Gardasil® target/probe	DNA sequence (22 bases)
Gardasil® HPV-11 L1 DNA[a]	CA**GA**TGATTA**C**CCC**A**A**C**AAATA-5′
INNO-LiPA HPV-11 probe	CA**A**G**T**GGTT**T**CCCC**A**G**C**AAATA-5′
Gardasil® HPV-18 L1 DNA[b]	TA**A**TTGATTA̲TGCCAGCA**GAT**A-5′
INNO-LiPA HPV-18 probe[c]	TAATTGATTATGCCAGCA**AA**C̲A-5′

[a] The 22-base interprimer segment of Gardasil® HPV-11 L1 gene DNA to the right can be amplified by the INNO-LiPA PCR primers designed for all common anogenital HPV DNA variants in this L1 region. However, the viral gene encoding the HPV-11 major capsid L1 protein for production of the Gardasil® HPV-11 VLPs is a synthetic construct which has a unique DNA sequence in this region with the interprimer location at 978 to 957 in the gene map depicted in GenBank Locus SCU55993. The 4 mismatched bases between the INNO-LiPA HPV-11 probe [54] and the Gardasil® HPV-11 in this region are underlined.

[b] The segment of the Gardasil® HPV-18 L1 gene DNA amplified by the INNO-LiPA PCR primers corresponds to a 22-base interprimer sequence from location 6573 to 6552 of the complete genome map of HPV-18, African type, as depicted in GenBank Locus EF202155.

[c] The INNO-LiPA HPV-18 probe [54] is designed to target a 22-base interprimer sequence shared by all HPV-18 European variants, e.g. GenBank Locus EF202148, and Asian-American variants, e.g. GenBank Locus EF202145. The two mismatched bases between the INNO-LiPA HPV-18 probe and the Gardasil® HPV-18 in this interprimer region are underlined.

There are numerous DNA sequence variations to the L1 gene within the genotype of HPV-18 which ultimately determines the amino acid composition of the major capsid protein of a virion. Phylogenetic analysis has shown that all HPV-18 isolates can be classified into 3 subtypes based on alignments of the DNA sequences of the variants, i.e. the European, the Asian-American and the African subtypes [57]. In Europe, it has been reported that all of the HPV-18 isolates from patients are found to be of the European or Asian-American variants [58]. In the U.S., 91% of the HPV-18 isolates from white women are reported to be of the European and Asian-American variants, and 64% of the HPV-18 isolates from African American women belong to the African variants [59]. Since the prevalence of the African variants of HPV-18 among European patients is negligible [58], the Dutch researchers who originally developed the HPV INNO-LiPA kit [54] naturally selected an HPV-18 probe targeting a homologous sequence shared by all European and Asian-American HPV-18 variants for the testing. However, the HPV-18L1 protein-coding gene chosen by the manufacturer for Gardasil® production is closely related to an African subtype [57,60]. Failure to detect a target sequence of an African variant HPV-18 DNA in the vaccine Gardasil® with a hybridization probe specifically designed for the European and Asian-American variant DNA may simply reflect the diversity of the L1 protein amino acid sequences within the genotype of HPV-18 (Table 2).

To reach the conclusion of finding unexpected HPV DNA residues in a vaccine product is a serious consideration. In addition to the extraordinary precautionary measures undertaken to avoid potential amplification of ambient HPV DNA sequences in conducting this research project as described in the Experimental section, the DNA sequence illustrated in Fig. 4 assures that the DNA template of this sequence has its origin in the Gardasil® vaccine because it represents a signature sequence of the HPV-11L1 gene synthetic construct specifically designed by the manufacturer for vaccine production. This HPV-11 DNA template does not exist in the environment and cannot come from any patient samples. In the author's laboratory serving a women population under the care of private gynecologists, HPV-18 is detected in about 6% of the routine HPV isolates [33], and about 80% of the HPV-18 isolates from the clinical samples belong to the European/Asian American subtype (author's unpublished data). All of the HPV DNA fragments detected in the vaccine samples other than those of the HPV-11 synthetic construct are of the African subtype of HPV-18. It is highly unlikely to contaminate the vaccine samples only with one HPV subtype so rarely encountered in the environment.

A major limitation of this study is that only 16 randomly selected Gardasil® samples have been tested. The findings presented in this report cannot prove that the Gardasil® vaccines being marketed other than these 16 samples also contain HPV-11 or HPV-18 DNA fragments; nor can the findings rule out the possibility that the Gardasil® vaccines on the world market may contain HPV-6 or HPV-16L1 gene DNA residues in addition to HPV-11 and HPV-18L1 gene DNA residues, or DNA residues from the plasmid pGAL110 and the yeast cells used in the production of the HPV VLPs.

The Gardasil® formulation with AAHS adjuvant has significantly increased the peak neutralizing antibody titers in vaccinated mammals based on poorly understood mechanisms [61]. The findings presented in this paper suggest that the residual HPV L1 gene DNA bound to a particulate aluminum mineral-based adjuvant in Gardasil® may activate the antigen-presenting cells or macrophages in an innate immunity reaction after endocytosis, which may have played a significant role in augmenting its immunogenicity. Co-delivery of a DNA vaccine and a protein vaccine with aluminum phosphate salts is known to stimulate a potent and multivalent immune response [62]. However, development of preventive human DNA vaccines is still at an experimental stage [63,64]. The potential risks of a DNA vaccine may include DNA integration into the host genome, induction of anti-DNA antibodies [65] and activation of the macrophages with release of cytokines, including tumor necrosis factor [27,66].

For Gardasil® vaccine production, the HPV DNA encoding the L1 capsid proteins is inserted into the plasmid pGAL110 to transform yeast cells for production of VLPs [5,67,68]. Possible expression systems also include mammalian cells [69]. Retention of residual recombinant DNA in protein-based vaccines has been a concern in the industry since induction of cancer is a single-cell phenomenon, and a single functional unit of foreign DNA integrated into the host cell genome might serve to induce cell transformation as a single event or part of a series of multifactorial events [70]. Chromosomal integration of foreign DNA may occur through poorly understood mechanisms [71,72] with uncertain consequences [73]. The short-term and long-term impact of the residual fragments of HPV L1 gene DNA or plasmid DNA if chemically bound to the mineral aluminum of AAHS nanoparticles is largely unknown and warrants further investigation.

5. Conclusion

Residual HPV L1 gene DNA fragments are present in the protein-based quadrivalent HPV vaccine. These DNA fragments are found to be firmly bound to the insoluble AAHS adjuvant particles. The clinical significance of these residual HPV DNA fragments bound to AAHS is not clear after intramuscular injection, and needs further investigation for vaccination safety.

Conflict of interest

Acknowledgments

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