Cross-neutralizing antibody titres against non-vaccine types induced by a recombinant trivalent HPV vaccine (16/18/58) in rhesus macaques

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ABSTRACT

Human papillomavirus (HPV) causes not only most cervical cancers but also cancers of the vagina, vulva, penis, anus, rectum, and oropharynx. Every year, 200,000 women die of cervical cancer in the world, and China accounts for about 10%. HPV vaccines are effective in preventing HPV infections thus HPV-related cancers worldwide. Studies on the clinical trials of the 2v Cervarix™ and the 4v Gardasil® have suggested that immunization with either of these vaccines provided some level of protection against other HPV types that are closely related to the types contained in the vaccines. Here we conducted a preliminary evaluation on the ability to induce cross-neutralizing antibodies in rhesus monkeys by a 3v HPV vaccine that targets HPV16, 18, and 58 and it is specifically designed for Chinese women. We found that this vaccine is no less than Gardasil® in terms of the ability to induce NAbs against non-vaccine types of HPV in rhesus macaques. These results provided evidence from the immunogenicity point of view that the KLWS 3v HPV vaccine is a strong competitor to the imported 2v and 4v HPV vaccines currently available on the market.

1. Introduction

Human papillomaviruses (HPVs) consisting of more than 100 genotypes can infect epithelial cells and cause genital warts or carcinomas in both males and females. HPV is classified into 5 genera: alpha, beta, gamma, mu, and nu, which can be further sorted into different species according to their genetic relatedness based on their L1 sequences [1–5] (Table 1). Members of the alpha genus are often associated with genital or mucosal carcinoma and skin warts. HPVs belonging to the α9 and α7 species (namely HPV16, 31, 33, 52, 58, 35, 18, 45, 59, and 39) contribute to almost 90% of cervical cancer [6]; HPV16 (the prototype of the α9 species) and HPV18 (the prototype of the α7 species) together are responsible for 70% of cervical cancer cases [7].

HPVs are non-enveloped double-stranded DNA viruses with a size of approximately 55 nm in diameter. The HPV genome has a region for the early genes E1-E7, a region for the late genes L1 and L2, and a non-coding region. The early genes are responsible for the viral life cycle and pathogenesis; whereas the late genes encode for the major capsid protein L1 and the minor capsid protein L2. The two late proteins together form the viral capsid in such a way that 72 copies of the L1 homo-pentamer form the viral external, and L2 sits in the centre of the L1-pentamers [4,5,8–12]. *In vitro*, the L1 protein can self-assemble into virus-like particles (VLPs) without the presence of the minor L2 protein. Such L1-VLP shares similar structures with the native virus, and induces high titres of neutralizing antibodies as it retains most of the neutralizing epitopes on the native virus [13–16]. These characteristics of the L1-VLP make it an ideal candidate for HPV vaccines.

There are currently four HPV vaccines available on the market. Cecolin® is a bivalent (2v) vaccine produced by INNOVAX that targets HPV16 and HPV18, Cervarix™ is also a 2v vaccine produced by GSK

Table 1
Classification of HPVs of alpha species.

Genus	Species	HPV type
Alpha	Alphapapillomavirus 1	HPV32, 42
	Alphapapillomavirus 2	HPV3, 10, 28, 29, 77, 78, 94, 117, 125, 160
	Alphapapillomavirus 3	HPV61, 62, 72, 81, 83, 84, 86, 87, 89, 102, 114
	Alphapapillomavirus 4	HPV2, 27, 57
	Alphapapillomavirus 5	HPV26, 51, 69, 82
	Alphapapillomavirus 6	HPV30, 53, 56, 66
	Alphapapillomavirus 7	HPV18, 39, 45, 59, 68, 70, 85, 97
	Alphapapillomavirus 8	HPV7, 40, 43, 91
	Alphapapillomavirus 9	HPV16, 31, 33, 35, 52, 58, 67
	Alphapapillomavirus 10	HPV6, 11, 13, 44, 74
	Alphapapillomavirus 11	HPV34, 73, 177
	Alphapapillomavirus 13	HPV54
	Alphapapillomavirus 14	HPV90, 71, 106
Beta	Betapapillomavirus 1-5	HPV5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47,49, 75, 76, 80, 92, 93, 96, 98, 99, 100, 104, 105, 107, 110, 111, 113, 115, 118, 120, 122, 124, 143, 145, 150, 151, 152, 159, 174, 182, 185, 195, 196, 198, 206, 209, 217, 227
Gamma	Gammapapillomavirus 1-27	HPV4, 48, 50, 60, 65, 88, 95, 101, 103, 108, 109, 112, 116, 119, 121, 123, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 144, 146, 147, 148, 149, 153, 155, 156, 157, 158, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 175, 176, 178, 179, 180, 181, 183, 184, 186, 187, 188, 189, 190, 191, 192, 193, 194, 197, 199, 200, 201, 202, 203, 205, 207, 208, 210, 211, 212, 213, 214, 215, 216, 218, 219, 220, 221, 222, 223,224, 225, 226, 228
Mu	Mupapillomavirus 1-3	HPV1, 63, 204
Nu	Nupapillomavirus 1	HPV41

Data were compiled based on "Reference genomes for Human papillomavirus" by Papillomavirus Episteme (https://pave.niaid.nih.gov/#explore/reference_ge nomes/human_genomes), and "Reference clones" by International Human Papillomavirus (HPV) Reference Centre (http://www.nordicehealth.se/hpvce nter/reference_clones/).
Numbers of the vaccine types are colored in bold black: HPV 16, 18, 58 for KLWS 3v vaccine, those of the non-vaccine types are colored in blue.

that targets HPV16 and HPV18, and the other two vaccines are developed by Merck: the quadrivalent (4v) Gardasil® is designed to prevent the oncogenic HPV16 and HPV18 as well as HPV6 and 11 that cause genital warts; whereas the nonavalent (9v) Gardasil® 9 can provide additional protections against HPV 31/33/45/52/58 compared with Gardasil®. All these four vaccines have been approved by the National Medical Products Administration (NMPA) in recent years. We have developed a recombinant trivalent (3v) HPV vaccine (which will be referred as KLWS 3v vaccine in this article) containing HPV16, 18, and 58 L1-VLP antigens (expressed in *Escherichia coli*) for Chinese women, as analyses on the type distribution of HPV infections in Chinese women revealed that the infection rate of HPV58 is higher compared to women in other countries, suggesting HPV58 is one of the common HPV types in China [17–32], and this vaccine has recently been approved by the NMPA for clinical trials.

It was hypothesized that due to the polyclonal nature of immune response, vaccination with HPV16 and 18 would induce neutralizing antibodies with broad spectra, thus provide at least some level of protection against other HPV types that are closely related to these two types [6]. The induction of cross-neutralizing antibodies by HPV16 and/or 18 L1-VLPs has been reported by several groups [33,34], and such cross-protection effect has been seen in clinical trials of both Cervarix™ and Gardasil® [35–42]. We conducted a preliminary evaluation of the induction of cross-neutralizing antibodies using sera of rhesus macaques from the Preclinical Safety Evaluation of our 3v HPV vaccine, aiming to shed some light on the cross-protection ability of this vaccine.

2. Materials and methods

2.1. Vaccine formulations

KLWS 3v vaccine contained HPV16/18/58 L1-VLPs and aluminum hydroxide in 500 μl acetic acid-sodium acetate buffer. 3 doses of KLWS 3v vaccine containing 60 μg/30 μg/30 μg/, 40 μg/20 μg/20 μg/and 20 μg/10 μg/10 μg/of HPV 16/18/58 L1-VLPs, respectively (termed high dose, middle dose and low dose of KLWS 3v vaccine). Each dose has 500 μg aluminum hydroxide. Gardasil was chosen as the positive control, which contained the same amounts of HPV16/18 L1-VLPs as middle dose KLWS 3v vaccine but less adjuvant (225 μg of aluminum hydrox-yphosphate sulfate per dose). They were intramuscular injection to groups of female rhesus macaques (n = 5) aged 3–5 in a 0, 4 and 24 week [43].

2.2. Source of serum

Serum samples of rhesus macaques collected in the Preclinical Safety Evaluation of KLWS 3v vaccine were used as the experimental material of this study. All animals were handled by licensed laboratory animal practitioners during the Preclinical Safety Evaluation, and were injected with ketamine and nembutal sodium for euthanasia at the end of the evaluation. Two weeks later after the third intramuscular injection at 24 weeks, serum samples were collected.

2.3. Pseudovirion-based neutralization assay (PBNA)

The pseudovirion-based neutralization assay (PBNA) was conducted essentially as described previously [44,45] with minor modifications. HPV type-specific pseudovirions (PsVs) encapsidating a green fluorescent protein (GFP) reporter plasmid were used for the PBNA. 293FT cells (Invitrogen, USA) were seeded in 96-well plates at a concentration of 15, 000 cells/well in complete DMEM medium and incubated at 37 °C for 4–6 h. HPV PsVs were diluted with complete DMEM medium to a concentration that the fluorescence expression level of cells in each well reached 15%. Serum samples were diluted with complete DMEM medium at a 4-fold dilution from 1:20 to 1:327,680. Equal volumes (60 μl) of HPV PsVs and serum samples were mixed and cooled at 4 °C for an hour, which were subsequently added to the cells. Following 72-h incubation at 37 °C, the plates were subjected to a SpectraMax MiniMax 300 Imaging Cytometer (molecular Devices, Sunnyvale, California, USA) to count the number of cells that were fluorescent. All tests were performed in duplicate.

2.4. Data analysis

The data were exported to Microsoft Excel, and the readings of each sample (including the PsV control) were averaged. The inhibition rate of fluorescence expression was calculated using the averaged value of each serum sample and that of the PsV control. The neutralization titre was determined by the Reed-Muench method as the final dilution factor that yielded 50% inhibition of fluorescence expression with PsVs alone, and reported as IC_{50}. The limit of quantification of the PBNA was set at 40 IC_{50}. Serum samples with neutralization titres equal or above 40 IC_{50} were considered positive; serum samples with neutralization titres below 40 IC_{50} were assigned a value of 1 for calculation purposes. Geometric mean titres (GMTs) and 95% confidence interval (95% CI) were calculated using GraphPad Prism version 5.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3. Results

HPV6, 11, 16, 18, 31, 33, 45, 52, and 58 PBNAs were performed on serum samples of rhesus macaques collected in the Preclinical Safety Evaluation of KLWS 3v vaccine. These rhesus macaques were negative
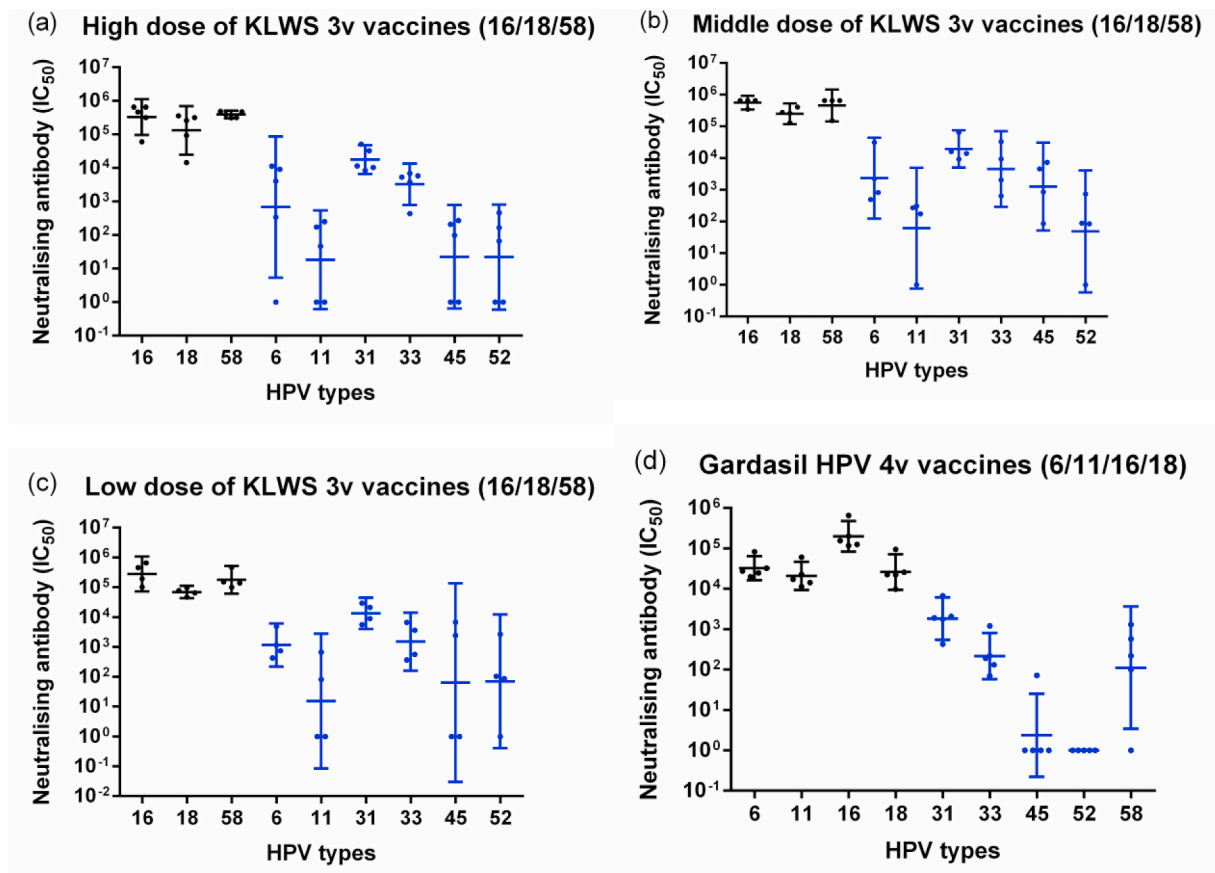
Fig. 1. Levels of HPV type specific neutralizing antibodies reported as IC_{50} in sera of rhesus macaques collected two weeks after the third immunization with (a) high dose, (B) middle dose, (c) low dose of KLWS 3v vaccines and (d) middle dose Gardasil®. GMT and 95% CI are shown as bars; IC_{50} values of each serum sample are indicated as dots. Bars and dots of the vaccine types are colored in black: HPV 16, 18, 58 for KLWS 3v vaccine and HPV 6, 11, 16, 18 for Gardasil®; those of the non-vaccine types are colored in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

for nine PBNAs before immunization. The serum samples were categorized into four groups based on the different dosages of either KLWS 3v or Gardasil® 4v HPV vaccines. Three dose levels of KLWS 3v vaccine were used, namely, high dose (1.5 vials per injection), middle dose (1 vial per injection), and low dose (0.5 vial per injection); and one dose level (1 vial per injection) of Gardasil® was used. All serum samples were collected two weeks after the third immunization with either KLWS 3v or Gardasil® HPV vaccines.

Two weeks after the third immunization, all four groups of rhesus macaques had neutralizing antibodies (NAbs) against vaccine types of HPV. The NAb levels in sera of the three groups of KLWS vaccinees were 10^5-10^6. In details, HPV18 NAbs of the low dose group was at the level of 10^5; HPV16, 18, 58 NAbs of the high and middle dose groups as well as the levels of HPV16 and HPV58 NAbs of the low dose group were all at the level of 10^6. For the serum samples of Gardasil® vaccinees, HPV16 NAbs were at the level of 10^6, and HPV6, 11, 18 NAb levels were 10^5 respectively (Fig. 1).

Serum samples of the three groups of KLWS vaccines showed different levels of NAbs against non-vaccine types (HPV6, 11, 31, 33, and 52). NAbs against HPV31 and HPV33 were at the level of 10^5 and 10^4 respectively, with all samples being positive. The levels of NAb against HPV6 were between 10^2 and 10^3.80% (4/5) of the serum samples in the high dose group were HPV6 NAb positive; whereas 100% (4/4) of the serum samples in the middle and low dose groups were HPV6 NAb positive. The levels of NAbs against HPV11 and HPV52 were the lowest (at the level of 10^1), and only 2 or 3 samples of each group were HPV11 or HPV52 NAb positive. The levels of NAb against HPV45 in samples of the high and low dose groups were 10^1, with 3 and 2 positive

samples respectively; whereas for the middle dose group, HPV45 NAb was at the level of 10^3, with all 4 samples being positive (Fig. 1).

For Gardasil® vaccines, different levels of NAbs against four (out of five) non-vaccine types of HPV (HPV31, 33, 45, and 58) were detected. NAbs against HPV31 and HPV33 were at the level of 10^3; levels of HPV33 and HPV58 NAbs were 10^2. One out of five samples was HPV45 NAb positive, with a titre of 72, and the other four samples were below the detection limit for HPV45 NAb. All five samples were below the detection limit for HPV52 NAb (Fig. 1).

4. Discussion

In this study, we evaluated and compared the NAb and cross-neutralizing antibody (cross-NAb) titres induced by KLWS 3v HPV vaccine (types 16/18/58) and Gardasil® in rhesus macaques. The results suggested that high, middle, and low dose of KLWS 3v HPV vaccines could induce not only high levels of NAbs against vaccine types (HPV16, 18, and 58), but also various levels of NAbs against non-vaccine types (HPV 6, 11, 31, 33, 45, and 52) in rhesus macaques. The control group, middle dose Gardasil® vaccine, induced high levels of HPV6, 11, 16, and 18 (the vaccine types) NAbs, and lower levels of HPV31, 33, 45, and 58 (non-vaccine types) NAbs. The levels of HPV16 NAbs induced by both vaccines were higher than those of HPV18 NAbs, which matches the findings of studies done on Cervarix™ and Gardasil® [34,46–51]. Unlike KLWS 3v HPV vaccine, Gardasil® did not induce HPV52 NAbs in rhesus macaques.

In terms of species, KLWS 3v HPV vaccine induced cross-NAbs against HPVs of the α9 (HPV31, 33, and 52) and α7 (HPV45) species.

In the sera of Gardasil® vaccinees, we detected cross-NAbs against HPV31, 33, and 58, which belong to the α9 species. The results suggest that different types of HPV of the same species share some epitopes. One thing worth noticing is that, the KLWS 3v HPV vaccine, which does not contain HPV antigens belonging to the α10 species, showed some level of cross-neutralization ability against HPV6 and HPV11 – the α10 species HPVs. Similar results were observed during a post hoc analysis on data of a Cervarix™ phase III PATRICIA (PApilloma TRIal against Cancer In young Adults) trial [52]. We therefore reached a preliminary conclusion from these results that the KLWS 3v HPV vaccine is no less than Gardasil® in terms of the ability to induce NAbs against both vaccine and non-vaccine types of HPV in rhesus macaques.

This is of great importance for Chinese women: in China, HPV vaccines are Class II vaccines that are currently not covered by the Social Medical Insurance System, and the three imported HPV vaccines currently available on the Chinese market (namely Cervarix™, Gardasil®, and Gardasil® 9) cost 2000–4000 RMB to complete the immunization procedure, meaning a population of approximately 0.7 billion belonging to the Low-income Group (defined as have an annual household income of less than 80,000 RMB (approx. 11,6000 USD)) would not be able to afford the cost of the vaccination. The utility of *Escherichia coli* as the expression host reduces the production cost of KLWS 3v HPV vaccine, making it a strong competitor to the imported HPV vaccines economically; and the findings of this study provided evidence from vaccine immunogenicity point of view for its strong competitiveness to vaccine of the same kind. Furthermore, KLWS 3v HPV vaccine has recently been approved by the CFDA for clinical trials, and its immunogenicity, especially the generation of NAbs against non-vaccine HPV types, and the cross-protection ability of this vaccine in humans will be analyzed in clinical trials.

Animal and human rights statement

The experimental animals were purchased from and fed in the Laboratory Animal Centre of the Academy of Military Medical Sciences (Beijing, China). The manipulation and vaccination on the animals were strictly referred to the guideline and compliant with the regulation, which was provided by the Laboratory Animal Centre of the Academy of Military Medical Sciences. Prior to the implementation, the experiment schemes and protocols were reviewed by Beijing Municipal Science and Technology Commission Administration Office of Laboratory Animals, and approved by Beijing Municipal Science and Technology Commission Laboratory Animal Management Ethics Committee. During the experiments, all animals were well-fed and monitored twice per day. All institutional and national guidelines for the care and use of laboratory animals were followed.

Author statement

Yan Wang and Yuying Liu conducted the experiment and analyzed the data, Yuying Liu also reviewed and edited the manuscript, Shutian Liang wrote the original draft, Fei Yin provided help during data analysis, Haijiang Zhang and Yongjiang Liu designed and supervised the study. All authors reviewed the manuscript.

Declaration of competing interest

All authors were employees of Beijing Health Guard Biotechnology Co., Ltd. when this study was conducted.

Acknowledgements

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