

Mouse acquired HPV tumor using dorsal skin-fold window chamber

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Human papillomavirus (HPV) plays important role in developing several types of cancer especially cervical cancer. In order to understand the viral pathogenesis, the animal model of HPV infection is very necessary. This communication reports establishment of an animal model carrying implanted HeLa cells, a human cervical cancer cell line via dorsal skin-fold window chambers. Nude mice were divided into 4 groups; each group contained different amount of HeLa cells, 2.5×10^5 , 5×10^5 , and 1×10^6 cells, and cell free medium (control), respectively. The results showed that even using the low number of HeLa cells (2.5×10^5), the tumor microvasculature was developed at 2 weeks after implantation with the enlarged tumor margin which then progressed to tumor mass in the following week. The existing tumor was confirmed to be HeLa-cell type by PCR, *in situ* hybridization, and HPV genotyping. By using linear regression analysis, it indicated that means of tumor size from each group significantly increased in relation to number of HeLa cells used ($R^2 = 0.98$, $y = 0.1171x + 4.35$). This mouse model will be useful for the further HPV studies particularly anti-cancer drugs efficacy.

Keywords: Dorsal skin-fold window chamber, HPV Tumor, Mouse model

Human papillomavirus (HPV) is a naked, icosahedral capsid, about 8 kb circular double stranded DNA virus, belonging to the family *Papillomaviridae*¹. At present more than 150 different HPV types have been reported². Approximately 40 types of HPV involve in anogenital tract infection and they can be classified as high risk (i.e., HPV 16, 18, 31, 33, and 35) and low risk (i.e., HPV 6 and 11) types according to the spectrums of their abilities to induce cancers^{2,3}. Unlike other viruses, HPV is highly host-specific, unable to propagate in normal cell culture and its replication cycle requires the differentiation process of keratinocytes^{2,4}. Thus, the interaction of virus and host is not well understood. Many attempts have been made using several means to develop animal model, starting from direct inoculation of HPV extracted from naturally human lesion to human neonatal foreskin and then grafted underneath the renal capsule of athymic mice⁵, direct transplant tumor tissue by xenograft technique and development of transgenic mice containing HPV E6 gene^{6,7}. An attempt has been made by inoculating HPV transformed cervical cell line, HeLa, into dorsal skin-fold window chamber. An animal model carrying

human cervical cancer cells was conducted. The implanted mice were monitored for their weight changes and size of tumor. The tumor microvasculature was also investigated under an intravital fluorescence videomicroscope. In addition, tumor lesion was then confirmed to be HeLa mass by using PCR technique, *in situ* hybridization, and HPV genotyping.

Materials and Methods

Animals—Female BALB/c-nude mice from National Laboratory Animal Center of Salaya Campus, Mahidol University, Bangkok, Thailand aged 4-8 weeks and weighing 20-25 g were used. The animals were handled as recommended by the guide for the care and use of experimental animals 1996, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, USA.

Cervical cancer cell line—HeLa cell is a human cervical cancer cell line which contains integrated HPV-18 genome approximately 10-30 copies/cell⁸. The cells were cultured in MEM medium (HyClone; USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, USA), penicillin (50 unit/ml) and streptomycin (50 µg/ml) with split ratio of 1:3. They were subcultured every 2-3 days regularly to

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maintain log phase. The cells were trypsinized into single cells before use.

HeLa-implantation in mice—Mouse was anaesthetized with pentobarbital sodium (50 mg/kg body weight, ip). The dorsal skin-fold window chamber was fixed on the dorsal of each mouse. The different amount of HeLa cells (2.5×10^5 , 5×10^5 and 1×10^6) were inoculated into the dorsal skin-fold window chamber. Each group contained 5 mice. Mice in the control group received only MEM medium. All the mice were regularly weighed every week continuously until 8 weeks and observed for the tumor development. The size of tumor mass was also measured.

Intravital fluorescence videomicroscopy—For the study of tumor angiogenesis, intravital fluorescence videomicroscopy was used. Two weeks after 1×10^6 HeLa-inoculation, the mice were anaesthetized with an ip injection of sodium pentobarbital (50 mg/kg). A catheter was inserted into a jugular vein for an application of fluorescence tracers [FITC-dextran, mol. wt=200,000, 0.5% (Sigma Chemical, USA)]. The tumor microvasculature was observed under an intravital fluorescence microscope (Nikon, Japan) equipped with a videocamera (Sony SIT68, Japan) and video-recorder (Sony SVT-124P, Japan). The $10 \times$ objective lens was used to observe microvessels within the tumor-bearing chamber. Based on the recorded videoimages, the tumor microvascular networks including neocapillary density were analyzed by using digital image processing software (Global Lab II).

Tumor size, tumor weight and histopathological examination—Tumor size was assessed using vernier calipers (VWR, St. Louis, MO) after the mice were anesthetized with an ip injection of sodium pentobarbital (50 mg/kg). An over dose of sodium pentobarbital was used for euthanasia the mice, tumor mass was carefully collected immediately, weighed and fixed into neutral buffer formalin (4% formalin, 0.4% NaH_2PO_4 , 0.65% Na_2HPO_4). Formalin-fixed, paraffin-embedded tissue samples were cut in 5 μm thick sections on a microtome with a disposable blade and fixed with Hematoxylin and Eosin (H&E) stains.

HPV-DNA detection—The tissue sections from tumor were extracted by using Qiagen DNA extraction kit (Qiagen, USA). The extracted DNA was then determined for the presence of HPV genome by polymerase chain reaction using specific HPV-L1 primers, L1C1; CGTAAACGTTTTCCCTATTTT-

TTT and L1C2; TACCCTAAATACTCTGTATTG⁹ and house keeping gene of human beta-globin, PC04; CAACTTCATCCACGTTCCACC and GH20; GAAGAGCCAAGGACAGGTAC¹⁰. Those primers were purchased from Invitrogen, Hong Kong. A reaction for each primer set was the same, i.e., 100 mM KCl, 20 mM Tris, 1.5 mM MgCl_2 , 200 μM dNTPs, 25 pM primer each, 1.25 units of Taq polymerase (Fermentas, Lithuania) and 1 μl of DNA sample in total volume of 50 μl . The amplification condition for HPV-L1 was 95°C, 10 min for a cycle, then denaturation at 95°C, 1.5 min, annealing 40°C, 1.5 min and extension 72°C, 2 min, repeated for 40 cycles followed by another extension at 72°C, for 10 min. The HPV-L1 product size was 250 bps. For human beta-globin, the amplification conditions was 94°C, 10 min for a cycle, then denaturation at 94°C, 30 sec, annealing 62°C, 1 min and extension 68°C, 1 min, repeated for 40 cycles followed by another extension at 68°C for 10 min. The amplified product size of human beta-globin was 268 bps. Detection of the amplicons was done by agarose gel electrophoresis.

HPV genotyping—DNA extracted from tumor cells was analyzed for HPV genotyping by using HPV genotyping kit (Inno-Lipa HPV Genotyping V2; Innogenetics; Germany). The principle of this technique is based on the reverse hybridization. A part of L1 region of the HPV genome was amplified and denatured biotinylated amplicons were hybridized with specific oligonucleotide probes which were immobilized as parallel lines on membrane strips. The procedure of test was performed as recommendation by company.

In situ hybridization of HPV-DNA—The presence of HPV-DNA in cells was detected by *in situ* hybridization using a probe specific to HPV-16/18 obtained from the Enzo Diagnostics kit (USA) in combination with the Dako GenPoint kit (Denmark). The system is colorimetric detection using tyramide reporter molecule to amplify the number of biotin which enhances binding of streptavidin-horseradish peroxidase (HRP) complex and 3,3' diaminobenzidine (DAB) was used as substrate. Then, the tissue was counterstained with Hematoxylin. The protocol was performed according to the manufacturer's recommendation. This assay was kindly performed by Professor Dr. Wasun Chantratita, Division of Molecular Virology, Department of Pathology, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand.

Results

Different amount of HeLa cells for establishing tumor mass—To evaluate how much the HeLa cells need to establish a tumor mass, various amount of HeLa cells (2.5×10^5 , 5×10^5 and 1×10^6) was inoculated into the dorsal skin-fold window chamber. The control group mice received MEM medium. Each group contained 5 mice. After inoculation, 3 mice died due to traumatizing lesion within 24 hr (Table 1). One was from group receiving HeLa 2.5×10^5 cells and another 2 mice were from group implanted with

HeLa 5×10^5 cells. The rest living mice were regularly weighed every week until 8 weeks. It was observed that HeLa cells at dose of 2.5×10^5 cells could generate tumor mass at 3 weeks after the inoculation, while it was only 2 weeks when the amount of HeLa cells were 5×10^5 and 1×10^6 cells (Table 1). Since the tumor size was enlarged up continuously, the chamber was removed when the tumor mass became larger usually at 3 weeks. At highest dose (1×10^6 cells), it was 100% successfully established tumor mass (Fig. 1). The linear regression analysis indicated that means of

Table 1—Tumor size and weight of inoculated mice
[Values are mean \pm SD]

No. HeLa cells No of mice with tumor (%)		Week (s) after implantation								
		0	1	2	3	4	5	6	7	8
2.5×10^5 4/5 (80)	Size (mm)	0	0	0	4.6 ± 1.70	8.0 ± 2.30	8.75 ± 1.5	9.25 ± 1.5	9.25 ± 1.5	10.25 ± 2.87
	Body weight (g)	23.24 ± 1.61	24.06 ± 0.64	24.26 ± 0.42	25.32 ± 1.23	26.16 ± 1.31	25.84 ± 1.24	25.82 ± 1.02	25.72 ± 1.07	26.36 ± 1.01
5×10^5 2/3 (66)	Size (mm)	0	0	4.00 ± 0.00	5.00 ± 1.00	5.3 ± 0.57	6.6 ± 0.57	7.3 ± 1.15	8.3 ± 1.52	9.6 ± 0.57
	Body weight (g)	18.6 ± 0.66	20.66 ± 1.15	21.60 ± 1.15	21.76 ± 1.07	21.83 ± 1.15	21.60 ± 0.26	22.03 ± 0.65	23.00 ± 0.72	23.73 ± 0.70
1×10^6 4/4 (100)	Size (mm)	0	0	3.0 ± 0.81	5.5 ± 1.00	6.5 ± 3.10	12.0 ± 6.53	14.25 ± 7.13	14.75 ± 7.36	14.75 ± 7.36
	Body weight (g)	17.3 ± 1.04	19.67 ± 0.88	20.60 ± 0.98	21.00 ± 1.37	21.85 ± 1.36	22.32 ± 1.14	22.37 ± 1.14	23.03 ± 1.31	24.02 ± 1.49

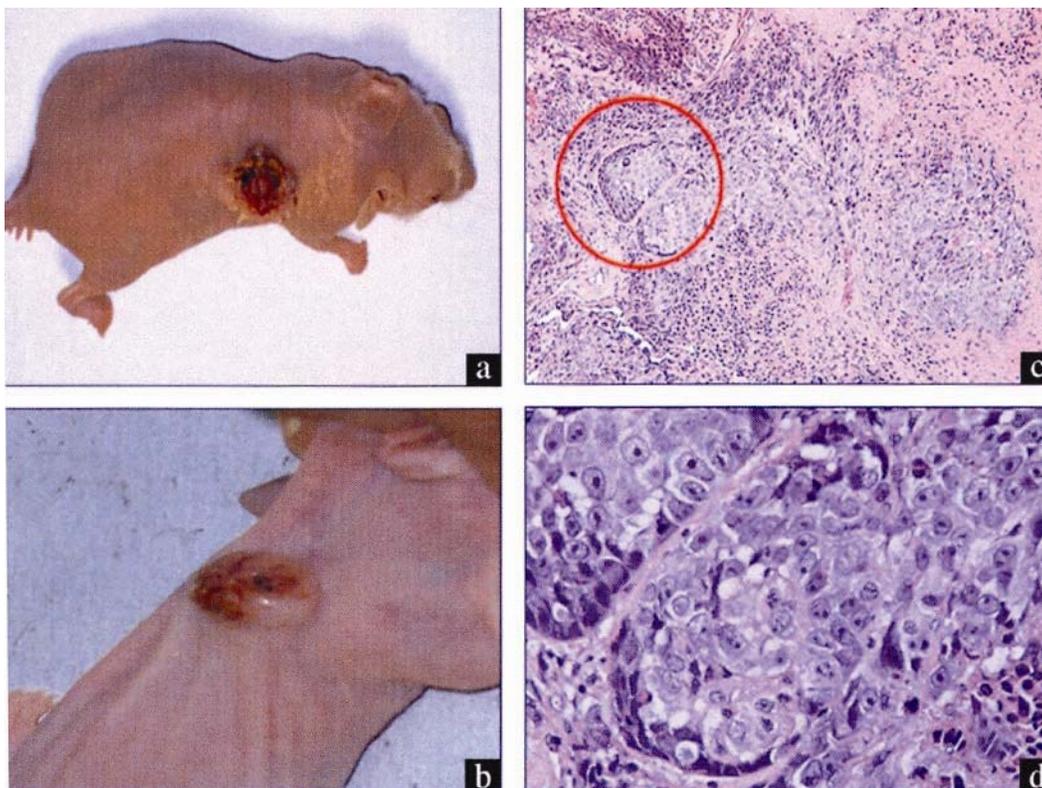


Fig. 1—Tumor growth at 2 weeks in mice implanted with HeLa cells 5×10^5 cells (a) and 1×10^6 cells (b). Tissue section of tumor from 2 weeks implanted mouse (1×10^6 cells) showed histopathology of squamous cell carcinoma (c) $\times 100$ and (d) $\times 400$.

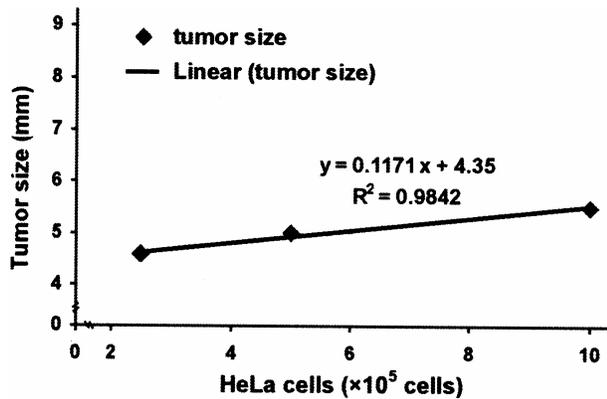


Fig. 2—Means of tumor size plotted against number of HeLa cells used

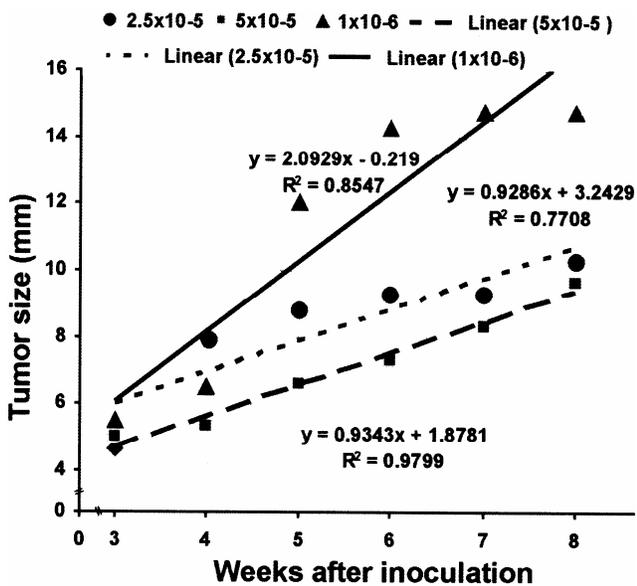


Fig. 3—Means of tumor size plotted against time intervals after the inoculations using different number of HeLa cells

tumor size from each group significantly increased in relation to number of HeLa cells used as shown in Fig. 2 ($R^2 = 0.98$, $y = 0.1171x + 4.35$). Figure 3 indicated that the rate of tumor growth increased in linear relation to the amount of HeLa cells used. In particular, it increased up to 2 times rate of tumor growth when the high number of inoculated cells was used, 1×10^6 cells.

Histopathological changes and tumor angiogenesis

—For studying histopathological changes and tumor angiogenesis, a new group of mice was inoculated with 1×10^6 HeLa cells. After 2 weeks of inoculation, 2 out of 5 mice from both control and HeLa-groups were sacrificed and observed for histopathological

changes and tumor angiogenesis. From H&E examination, the characteristic of squamous cell carcinoma was found in 2-week HeLa-mice whereas none was present in control mice (Fig. 1). In addition, the appearance of developed tumor on dorsal skin of those mice also showed necrosis and hematoma lesions. Moreover, by using intravital fluorescent videomicroscopy, the results of tumor micro-circulation revealed the increase in neocapillary density in 2-week HeLa group as compared to control group (Fig. 4). One mouse was sacrificed at 3 months for histopathological study which also showed the same characteristic of squamous cell carcinoma. The other 2 mice formed grossly identifiable tumors were able to live up to 6 months.

HPV detection—In order to confirm that tumor growth came from HeLa cells, DNA extraction from tumor tissue was amplified with HPV-L1 specific primers. Moreover, tissue sections were determined by *in situ* hybridization with probe specific to both HPV-16 and 18. The dark brown staining cells were HPV DNA positive cells (Fig. 5c). Finally, HPV genotype was done by using HPV genotyping kit (Inno-Lipa HPV Genotyping V2; Innogenetics; Germany). All assays indicated that HPV-DNA of type 18 was found in the extracted DNA and located in the tumor cells (Fig. 5).

Discussion

This present results demonstrated the application of dorsal skin-fold window chamber technique to assist in developing an *in vivo* model for HPV acquired tumor. The direct inoculation of human cervical cell line (HeLa) into dorsal skin-fold window chamber could induce the tumor growth and became a tumor mass (Figs 1 and 2). The histopathological examination also confirmed that the human cervical cancer cells can grow in mice tissue, in particular with the characteristic of squamous cell carcinoma (Fig. 1).

Different amount of HeLa cells were used and the results indicated that: (i) the size of tumor increased significantly in relation to the amount of transplanted cells, and (ii) the rate of tumor growth (size/time duration) linearly increased in all 3 doses of HeLa cells (Fig. 3). The results of intravital fluorescent videomicroscopy (Fig. 4) also indicated that at the site of implantation, the important process of tumor angiogenesis was existed within 2 weeks similar to other types of tumor implantation models¹¹. The longest duration for these transplanted mice to stay

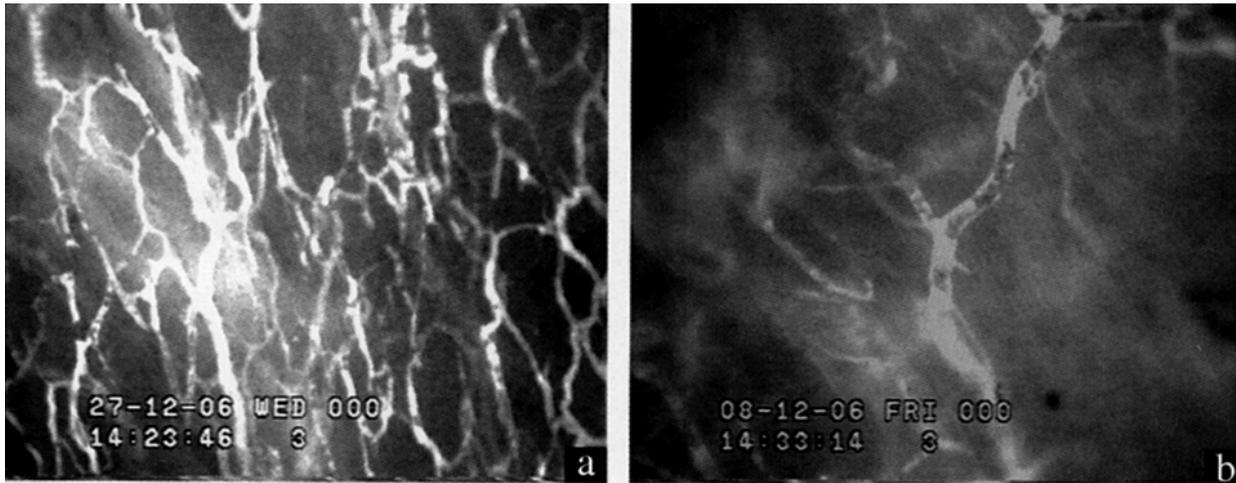


Fig. 4—Intravital Fluorescence Videomicroscopy of 2 week- 1×10^6 HeLa implanted mouse (a) compared with control mouse (b) $\times 100$

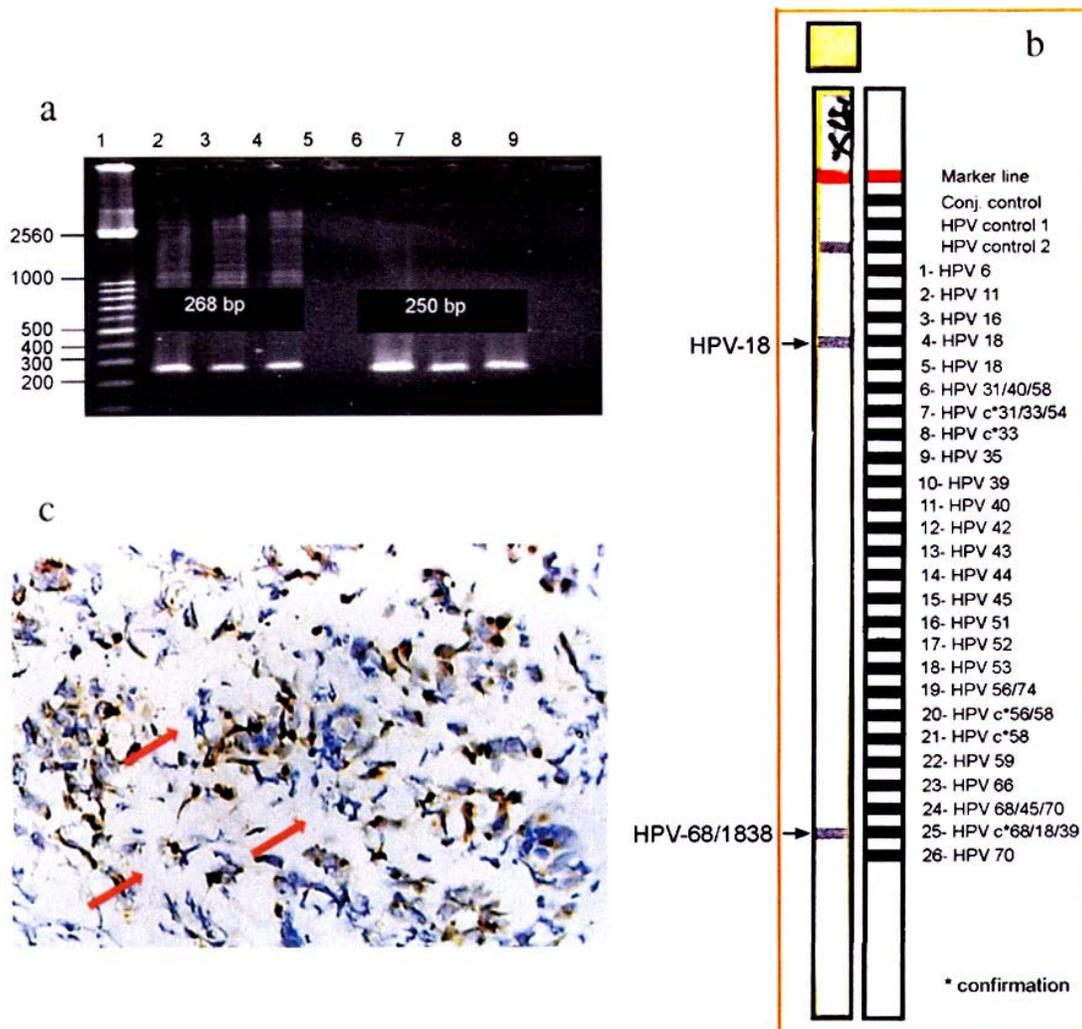


Fig. 5—HPV detection in tumor tissues by (a) PCR, Lane 1: DNA marker (bp); Lane 2 and 6: HeLa DNA; Lane 3, 4, 7 and 8: tumor extracted DNA; Lane 5 and 9: normal mouse tissue; Different set of primers was done; Lane 2, 3, 4, 5: human beta-globin primers, Lane 6, 7, 8, 9: HPV-L1 primers. (b) HPV genotyping (c) *In situ* hybridization (arrow indicated positive HPV-DNA inside the cells)

alive was about 6 months after the inoculation. Moreover, the developed tumor was definitely caused by HeLa cells growth (Fig. 5).

Although several studies reported the development of animal models with HPV infection^{12,13}, the methods used still have a lot of limitation and not practically done. In short, (i) the difficulty in the preparation of purified HPV viruses from infected tissue, (ii) the need of human tissue as the starting materials used for viral inoculation before transplanting it into an animal and (iii) the beginning steps of tumor establishment such as increase vascularization or angiogenesis could not be observed in details. The advantages of the dorsal skin-fold window chamber technique in developing mouse bearing HPV tumor are benefit for the future study on testing any tumor-growth inhibitors especially anti-angiogenesis drug. Moreover, the linear regression line will make it simple to compare the efficacy of those inhibiting agents. However, this technique always developed the necrotizing area which appeared as hematoma lesion but it is similar to previously reports of another xenograft techniques¹⁴.

In conclusion, the present novel *in vivo* model for HPV acquired tumor is simply and highly reproducible in term of tumor induction model. This kind of animal model may be useful for screening and selecting candidate of novel anti-cancer agents in the future.

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