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Citation for final published version:

Yang, Yang, Wang, Hexiao, Zhang, Xinrui, Huo, Wei, Qi, Ruiqun, Gao, Yali, Zhang, Gaofeng, Song, Bing, Chen, Hongduo and Gao, Xinghua 2017. Heat increases the editing efficiency of human papillomavirus E2 gene by inducing upregulation of APOBEC3A and 3G. *Journal of Investigative Dermatology* 137 (4), pp. 810-818. 10.1016/j.jid.2016.06.635

Publishers page: <http://dx.doi.org/10.1016/j.jid.2016.06.635>

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**Heat increased the editing efficiency of human
papillomavirus E2 gene by inducing upregulation of
APOBEC3A and 3G**

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Short title: Editing of HPV by APOBEC3

Abbreviations: APOBEC3, apolipoprotein B mRNA-editing catalytic polypeptide 3;
HPV, human papillomavirus; CA, condyloma accuminatum; IFN, interferon

Abstract

Apolipoprotein B mRNA-editing catalytic polypeptide (APOBEC) 3 proteins have been identified as potent viral DNA mutators and have broad antiviral activity. In this study, we demonstrated APOBEC3A (A3A) and A3G expression levels were significantly upregulated in HPV infected cell lines and tissues. Heat treatment resulted in elevated expression of A3A and A3G in a temperature-dependent manner in HPV infected cells. Correspondingly, 44 °C heating treated HPV infected cells showed accumulated G-to-A or C-to-T mutation in HPVE2 gene. Knockdown of A3A or A3G could promote cell viability, along with the lower frequency of A/T in HPV E2 gene. In addition, regressing genital viral warts also harbored high G-to-A or C-to-T mutation in HPVE2 gene. Taken together, we demonstrate that APOBEC3 expression and editing function was heat sensitive to a certain degree, partly explaining the mechanism of action of local hyperthermia to treat viral warts.

Key words: heat, hyperthermia, condyloma acuminatum, APOBEC3, hypermutation, HPV

Introduction

Human papillomavirus (HPV) is a group of small, nonenveloped and double-stranded DNA viruses found in a variety of proliferative and some malignant lesions of epithelial origin (Shterzer *et al.*, 2014). HPVs are the primary etiological agents of several clinical entities (Giannaki *et al.*, 2013). Condyloma acuminata (CA) is a common sexually transmitted HPV infection in the external genital and perianal areas. Local hyperthermia has been successfully used in the treatment of HPV-infected skin lesions, with cure rates ranging from 41% to 93.5% according to differences in hyperthermia conditions, therapeutic procedure and responsiveness of specific conditions (Ma *et al.*, 2012). It has been presumed that local hyperthermia can directly influence virus activity, and promote host immune responses to eliminate HPV infections (Li *et al.*, 2009; Ma *et al.*, 2012; Wang *et al.*, 2010; Zhu *et al.*, 2010).

Apolipoprotein B mRNA-editing catalytic polypeptide (APOBEC) deaminases are a family of protein that edit DNA and/or RNA sequence, which include activation-induced cytidine deaminase (AID), APOBEC1, -2, -3A, -3B, -3C, -3DE, -3F, -3G, -3H and -4. Depending on cell type and tissue environment, the expression and localization of APOBECs are different (Moris *et al.*, 2014; Vieira and Soares, 2013). APOBEC family displays deteriorating genetic information of many viruses by hypermutation-dependent and -independent approach. Recently, several high-profile

studies suggested that APOBEC3-mediated mutagenesis is highly enriched in HPV-positive cervical and head-and-neck cancers (Burns *et al.*, 2013; Roberts *et al.*, 2013). In addition, two human APOBEC3A (A3A) and A3G gene are expressed in epithelial cells and both of them are reported to be involved in editing HPV by inducing cytidine (C) to uracil (U) hypermutation (Vartanian *et al.*, 2008; Wang *et al.*, 2014).

Interferons (IFNs) and LPS could positively regulate A3 expression by different pathways (Mehta *et al.*, 2012). The expression of A3 has been determined based on mRNA levels in cells, using quantitative PCR (Koning *et al.*, 2009; Refsland *et al.*, 2010). While an extremely sensitive PCR method, differential DNA denaturation PCR (3D-PCR), has detected hypermutations from C to T or G to A (Kukimoto and Muramatsu, 2015). Here, we detected the expression of A3A and A3G in HPV infected cells, and further tested whether local hyperthermia could affect A3A and A3G expression in HPV infected cells, and subsequent gene editing of susceptible HPV genes.

Results

Expression of A3A and A3G is elevated in CA than in normal skin

By immunohistochemical staining, we observed that A3A was slightly expressed in keratinocytes from normal skin; however, there was much stronger expression of A3A in CA tissues than in normal skin (Figure 1a). A3G was either very weak or negative in normal skin; however, a strong expression of A3G was observed in most of CA samples (Figure 1b). The mean optical density of A3A and A3G in CA was higher than normal skin tissue by analysis with Image-Pro plus software.

Heat/Hyperthermia induced up-regulation of A3A and A3G in HPV harboring cells and the skin

Five cervical cell lines were used. C33A and HT3 are negative for HPV, SiHa harbors 1-2 copies of HPV16, CaSki harbors about 60-600 copies of HPV16 and HeLa harbors HPV18 (Baker *et al.*, 1987; Ostrowska *et al.*, 2010; Pattillo *et al.*, 1977; Yee *et al.*, 1985; Choi *et al.*, 2005). As shown in Figure 2a and b, hyperthermia at 42 °C and 45 °C induced 7- and 14- fold increase of A3A mRNA, and 14- and 60- fold increase of A3G, respectively, in CaSki cells. Hyperthermia at 42 °C and 45 °C also induced 3-4 fold increase of A3A and A3G mRNA in SiHa cells. Hyperthermia on C33A cells, however, did not induce any significant change of A3A and A3G. Western blotting confirmed the significant elevated expression of A3A and A3G in CaSki cells,

moderately elevated expression in SiHa cells and no obvious changed expression in C33A cells (Figure 2c). The expression of A3A and A3G induced by hyperthermia in HeLa and HT3 cell lines was presented in Supplementary Figure S1a-b.

We collected 11 surgically removed samples from CA patients and divided each sample into equal three parts. Each part from a patient was subjected to hyperthermia at 37 °C, 42 °C and 45 °C for 30 min as described (Ostberg *et al.*, 2003; Wang *et al.*, 2009; Zhu *et al.*, 2010). Real-time PCR analysis showed that there was a significant increase in the production of A3A and A3G transcripts in CA tissue subjected to local hyperthermia. There was a 2-3 and 11-13 fold-increase of A3A transcripts in CA tissues subjected to hyperthermia at 42 °C and 45 °C, respectively, as compared with that at 37 °C (Figure 2d); the increase of A3G transcript level was 4-6 and 10-12 fold when subjected to hyperthermia at 42 °C and 45 °C, as compared to that of 37 °C, respectively (Figure 2e). The expression levels of A3A and A3G remained stable in normal skin, regardless of hyperthermia temperature employed.

Taken together, hyperthermia further elevated the expression levels of A3A and A3G in HPV infected cells, and the effects appeared in a temperature- dependent. It is also demonstrated that cells with high HPV copy number had stronger A3A and A3G expression when subjected to hyperthermia.

Heat induced hypermutation of E2 gene in cervical cancer cells

A3 proteins have been identified as a restriction factor against HPV infection by editing viral DNA (Kukimoto *et al.*, 2015; Warren *et al.*, 2015). We investigated whether overexpression of A3A and A3G induced by heat in cervical cancer cells could mutate HPV E2 sequence. First, we applied 3D-PCR assay to detect the present of G→A or C→T hypermutation by decreasing the denaturation temperature. The mutation of E2 gene could undermine E2 gene functions, directly or indirectly affecting development and final outcome of HPV-associated diseases (McBride, 2013; Xue *et al.*, 2015a). The E2 genes from CaSki cells subjected to heat at 44 °C were amplified at a relatively lower denaturation temperature (83.7 °C) than those at 37 °C (85.5 °C). As with CaSki cells, SiHa cells heat-treated at 44 °C yielded the PCR products at a lower denaturation temperature (84.1 °C) compared to those without undergoing heating (85.5 °C). The results indicated that mutation frequency in CaSki cells was probably higher than that in SiHa cells after heating at 44 °C (Figure 3a).

To detect the presence of E2 hypermutation, amplified E2 fragments at the lowest denaturation temperature were excised from gels and inserted into a T vector for sequence determination. Sequence analysis revealed CaSki cells and SiHa cells treated with heat exhibited a robust preference for C to T or G to A hypermutation in the coding-strand of HPV E2 gene. Amplified E2 gene carried 12 G to A mutations, 18 C to T mutations in CaSki cells, and 9 G to A mutations, 12 C to T mutations in SiHa cells, respectively (Figure 3b). E2 DNA amplified and sequence analysis in HT3 and HeLa cells treated or untreated with heat were presented in Supplementary Figure

S1c-d. These results collectively demonstrated that heat boosted- high- expression of A3A and 3G contributed to E2 hypermutation in cervical cancer cells. It was intriguing to note that E6 hypermutation was not significantly different among the treated cell lines (data not shown), while if other open reading frames of HPV are susceptible remains further investigation.

Regressing CA lesions had elevated mRNA expression of A3A and A3G

We previously reported local hyperthermia at a small part of an extensive CA patient resulted in clearance of all the lesions. During the regression of lesions, there was dense infiltration of CD8+T cells as well as CD4+T cells at the untreated lesional sites, 2-4 weeks post initiation of the treatment. Their number returned to near normal value at week 6 (Huo *et al.*, 2013). Following similar protocol as reported (Huo *et al.*, 2013), we took serial biopsies specimens from six patients with multiple CA lesions, who received five sessions of local hyperthermia at 44 °C for 30mins each, at one part of the lesions. Each of the patients we collected was recorded his/her gender, age, number of warts and HPV subtype (Supplementary Table S1). Biopsies were taken on untreated area before and 2-, 6- weeks after the first treatment. Four of the patients showed signs of clinical regression at week 6 post treatment, one patient (P4) had all her lesions resolved at week 4. As shown in Fig.4a and b, the mRNA expression of A3A and A3G increased during the regression course of the lesions. In the case of the patient who failed to respond to hyperthermia treatment clinically, we did not detect significant changes of mRNA expression of A3A and A3G at the three time points

(P6).

Among the five patients who responded to treatment, 3D-PCR showed that CA samples during the regression course yielded the PCR products at a lower denaturation temperature compared to those samples prior to treatment. At the late time(6 weeks) of CA regression, denaturation temperature further decreased, ranging from 84.9 °C to 84 °C, suggesting the existence of more extensive A/T hypermutation (Figure 4c). DNA sequencing of the E2 fragments revealed that hypermutation in the hyperthermia treated samples was biased toward C to T mutations and G to A mutations (Figure 4d). What's more, patient No. 4 (P4) who showed quick clinical response possessed more mutations of E2 gene (Figure 4 e). The patient who failed to respond clinically had no obvious biases toward C to T and G to A conversions. The association of hyperthermia treatment response, HPV typing and E2 hypermutation is an interesting question for further investigation.

Inhibition of A3A or A3G decreased the proportion of A/T in E2 gene

To investigate the effect of A3A and A3G on the mutation frequency of E2, we knocked down the gene using two short-interfering RNA (siRNA) to downregulate A3A or A3G expression. Real-time PCR and western blot showed significant decrease in mRNA and protein levels after transfection compared with scrambled siRNA (Figure 5a-b). In subsequent experiments, CaSki and SiHa cells were pretreated with different agents (Scrambled siRNA, A3A-siRNA1, A3A-siRNA2, A3G-siRNA1, or A3G-siRNA2) for 72 hours, total DNA was extracted and 3D-PCR was performed.

Compared with control and scrambled siRNA (85.5 °C), 3D-PCR products were recovered at a higher temperature (86 °C-87 °C) in the A3A/G-silenced cells (Figure 5c). Sequencing of cloned 3D-PCR products revealed that CaSki cells and SiHa cells knocking out A3A or A3G produced a relatively low A% and T% in E2 fragments, indicating that A3A and 3G contributed to yield hyperedited HPV E2 genomes.

Inhibition of A3A or A3G promoted the viability of cervical cancer cells

The effect of A3A and A3G on the viability of cervical cancer cells was measured by MTS assay from day 1 to day 4. Compared to transfection with scrambled siRNA, cell viability was increased with A3A or A3G knockdown at day 3, and persisted to day 4 in CaSki cells and SiHa cells. While there was no obvious statistical difference of cell viability in C33A cells, either transfected with A3A/G siRNA or not (Figure 6). These results indicated that A3A and A3G could regulate the cell viability of HPV infected cervical cancer cells.

Discussion

APOBEC proteins represent a family of cytosine deaminases to resist viral infection. During replication, double-stranded DNA viruses may be susceptible to deamination yielding G-to-A and C-to-T substitutions (Suspene *et al.*, 2005). Data from several laboratories have documented A3 induced hypermutation signatures in multiple cancers, including HPV-associated cervical tissues in early stages of cancer progression and HPV positive head-and-neck squamous cell carcinomas and decreased HPV infectivity (Henderson *et al.*, 2014; Warren *et al.*, 2015). A3A and A3G are expressed in epithelial cells, where HPV has been shown to replicate and reside (Vartanian *et al.*, 2008). However, information regarding the involvement of A3A and A3G in HPV-associated proliferative dermatologic diseases is limited.

It was reported that hyperthermia induce a significant increase of IFN in CA (Zhu *et al.*, 2010) and IFN subsequently could positively regulate APOBEC3 expression (Mehta *et al.*, 2012). A3 was reported to reduce the infectivity of HPV16 pseudovirion (Ahasan *et al.*, 2015). Our previous study suggested that local hyperthermia at 44 °C in a defined protocol was an effective method to treat cutaneous warts (Huo *et al.*, 2010). Local hyperthermia could enhance the production of IFNs and increase the apoptotic index of HPV infected skin, partly explaining its effectiveness to treat the condition ((Wang *et al.*, 2010; Zhu *et al.*, 2010). One of the advantage of local hyperthermia treatment against HPV infected skin is that patients

with multiple lesions tended to experience clearance of all the lesions, once the targeted lesion responded, a phenomenon highly suggestive that local hyperthermia aided in the establishment of specific immune response against HPV infected skin. Indeed, it was observed that untargeted resolving lesional skin had abundant CD4 and CD8+ T cell infiltration (Huo *et al.*, 2013). In the present study, we demonstrated that heating/hyperthermia induced A3A and A3G expression in HPV infected cells, and their elevated expression was functional to the effect that higher number of HPV E2 hypermutation was detected. Unexpectedly, hypermutation in HPV E6 was not induced (data not shown). We postulate that at least specific genetic sequence may render their reactivity with APOBEC3. To our knowledge, HPV LCR DNA was not able to be edited by APOBEC (Wang *et al.*, 2014). Detection of other HPV open reading frames hypermutation by APOBEC3 is a good topic for further investigation.

Though E2 gene constitutes only a small part of the total viral genome, E2 plays an important role in replication and transcription of HPV by binding to specific cognate sequences in the viral genome (Sunthamala *et al.*, 2014). Even at relatively low levels, the expression of E2 could maintain the cells in a condition compatible with viral DNA replication, even induce genomic instability and promote carcinogenesis in HPV- associated cervical lesions (Xue *et al.*, 2015b). E2 protein may have a great influence on the progression of diseases. Our previous work indicated that overexpression of A3A could produce more A/T mutation frequencies of HPV E2 gene (Oncology Letter, accepted). On the contrary, insufficient A3A

resulted in lower A/T mutation frequencies of HPV E2 gene. So we speculate that APOBEC3-induced hypermutation of E2 is another mechanism of action that hyperthermia took effect to treat HPV infected skin. We detected HPV E2 gene mutation in different samples (including cell lines and tissue samples) and found that APOBEC edited E2 gene regardless of HPV subtypes. It tends that hyperthermia treatment induced stronger APOBEC3 expression and higher E2 mutation rates in tissue blocks with low risk HPV types than those with high risk HPV types. A large scale clinical trial is required to examine whether HPV types affect the efficacy of hyperthermia therapy. Whether hyperthermia could directly induce A3 expression is a matter of further investigation. As hyperthermia could induce the production of IFNs, and IFNs are known to induce A3 (Payne *et al.*, 2000), an indirect effect of hyperthermia on the A3 expression could not be excluded. Regression of HPV infected skin is accompanied with dense T cell infiltration. Regressing CA lesions had high expression of A3 and high HPV E2 hypermutation. How infiltrating T cells correlate with E2 hypermutation is another interesting topic to be further investigated.

Materials and Methods

Clinical Samples

Histological evaluation of A3A and A3G proteins were performed on 10 archived, formalin fixed and paraffin embedded, histologically confirmed CA tissue blocks at the Department of Dermatology, No.1 Hospital of China Medical University. Five samples of normal adult skin tissues were collected as controls.

Genital samples for *in vitro* study were discarded specimen from 11 patients (3 males and 8 females) with clinically diagnosed CA and who were treated surgically. They had not received any prior anti-wart treatments, and no documented systemic disease(s). Five foreskin specimens from patients undergoing genital plastic surgery were obtained as normal controls. Serial CA samples were from six CA patients (2 male and 4 females) with multiple lesions (≥ 4), again they had not received any prior anti-wart treatments, and no any documented systemic disease(s).

This protocol of collecting human samples was approved by Institutional Review Board and was conducted in conform to the Declaration of Helsinki. All samples were obtained with informed consent.

Cell culture and hyperthermia

Human cervical cancer cell lines (C33A, SiHa, CaSki, HeLa and HT3) were purchased from ATCC (Manassas, VA) and were cultured as recommended. The

C33A, SiHa and HT3 cells were maintained in MEM (HyClone, Shanghai, China) medium supplemented with 10% fetal bovine serum (FBS, Biological Industries, CT, USA). The CaSki and HeLa cells were cultured in RPIM-1640 medium (HyClone, Shanghai, China) with 10% FBS. All cell lines were incubated at 37°C in a humidified incubator with 5% CO₂.

Cells were seeded into 6-well plates and cultured overnight before being treated with hyperthermia at 37°C, 42°C and 45°C for 30min, then were cultured for another 12h or 24h and were collected for subsequent RNA and protein testing.

Immunohistochemical study of A3A and A3G

4 µm paraffin-embedded tissue sections were dewaxed in xylene, rehydrated in alcohol, and then incubated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by heating pressure cooker in 0.01 mol/L citrate buffer (pH, 6.0). The tissue sections were then incubated with A3A monoclonal rabbit antibody (dilution 1:1000; Abcam, Cambridge, MA) and A3G monoclonal rabbit antibody (dilution 1:200; Abcam) overnight at 4 °C. After rinsing in phosphate-buffered saline (PBS), sections were incubated with biotinylated secondary antibody at room temperature for 30 min. Subsequently, sections were visualized with the EnVision plus detection system followed by counterstaining with hematoxylin. Image-pro Plus software was used to assess the mean optical density.

Heat/Hyperthermia to treat cells or tissues

A skin organ culture protocol was used in the study (Ostberg *et al.*, 2003) and the procedure for local heating has been previously described for vitro experiment (Wang *et al.*, 2009; Zhu *et al.*, 2010).

In regard to *in vivo* study, six CA patients with multiple lesions received hyperthermia treatment at 44 °C once a day for 3 consecutive days, with each treatment session lasting 30 min. A week later, patients subjected to the similar treatment for 2 consecutive days, using the hyperthermia device as described (Huo *et al.*, 2014; Huo *et al.*, 2013) We chose the biggest lesion in size as therapeutic target. Then, the patients were followed-up biweekly to six weeks. Serial biopies were taken on untreated lesions, before treatment, 2 weeks and 6 weeks after the first treatment. The specimens were embedded in Tissue-Tek[®] (OCT compound, Sakura, USA) and stored at -80 °C for further analyzes.

Western Blot

After treatment, the cells were washed twice with PBS and lysed with 80µl RIPA buffer (Pierce, Rockford, USA), and 1×complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) on ice for 10min. An aliquot of the total protein (30 µg) was loaded into each lane in a 12% sodium dodecyl sulfate polyacrylamide gel and then electrophoretically transferred to polyvinylidene difluoride membranes. The membrane was blocked with 5% nonfat milk in 0.01M TBS (pH 7.4) and 0.05% Tween-20 (TBST) at room temperature for 1 hour before incubation with either anti-A3A or A3G antibody at 4 °C overnight. After washing, the membranes was

incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Proteintech, Wuhan, China) for 2 hours at room temperature and the bands were visualized with ECL Western Blotting Substrate (Pierce).

RNA isolation and real-time PCR

Total RNA was extracted by using miRNeasy mini kit (Qiagen, Stockach, Germany) and reverse-transcribed with PrimeScript™ RT reagent kit (TaKaRa, Ohtsu, Japan). Real-time PCR was performed to calculate relative mRNA expression level using primers A3A fwd, 5'-GAAGGGACAAGCACATGGAAGC-3', and A3A rev, 5'-ATCTACTTGATCGGGAGCATAAC-3', A3G fwd, 5'-GGTGTATTCCGAACTTAAGTAC-3', and A3G rev, 5'-CAAGGAAACCGTGTTTATGTGG-3', GAPDH fwd, 5'-AAGAGCACAAGAGGAAGAGAGAGAC-3', and GAPDH rev, 5'-GTCTACATGGCAACTGTGAGGAG-3'.

RNA isolation and real-time PCR

Two siRNAs specific for A3A (A3A siRNA1 and 2) and two siRNAs specific for A3G (A3G siRNA1 and 2) as well as scrambled sequence (Scrambled siRNA) were purchased from Life Technologies Corporation (Foster City, CA, USA). The siRNA sequences are listed in Supplementary Table S2. Cells were seeded the day before transfection in antibiotic-free Medium and transfected with Silencer® Select A3A siRNA or A3G siRNA and mixed with Lipofectamine™ RNAiMAX (Thermo Fisher

scientific, USA) as transfection reagent according to the manufacturer's instructions.

After transfection, cells were assessed for changes in mRNA and protein levels.

Detection of hypermutation in HPV E2 gene

Genomic DNA was extracted from specimens using DNeasy blood and tissue kit (Qiagen) according to the supplier's instructions. The HPV E2 gene was detected by 3D-PCR with GoTaq Colorless Master Mix (Promega, Mannheim, Germany) and specific primers as previously described (Wang *et al.*, 2014). The initial PCR condition was denaturation step at 94 °C for 4 min, followed by 35 cycles, each denaturing at 94 °C for 16s, annealing at 55 °C for 20s, extension at 68 °C for 50s, and an additional 10 min extension step at 65 °C. Second-round PCR was performed as follows: 85.9-84 °C for 5 min, followed by 35 cycles of denaturation at 85.9-84 °C for 45s, annealing at 50 °C for 30s, extension at 65 °C for 38s and a final elongation step at 65 °C for 10 min. To analyze the site-specific hypermutation frequency, PCR products from 3D-PCR were cloned into T vector and successful recombinant clones were selected randomly for further sequencing.

MTS assay

The cells transfected with A3A-siRNA or A3G-siRNA were seeded in 96-well plates at a density of 8000 cells (100µl) per well and at day 1, 2, 3 and 4, 20 µl of MTS (Promega, Madison, WI) was added to the medium and then continued incubation at 37 °C for 3 hours. The cell viability was analyzed by measuring the

absorbance at 490nm using a spectrophotometer.

Statistical analysis

All experiments were repeated at least three times and statistical analyses were performed using GraphPad Prism (GraphPad software, San Diego, CA). For more than two groups of experiments, the differences between each group were analysed by one-way analysis of variance (ANOVA). Quantitative results were expressed as mean \pm SD. For experiments with only two groups, unpaired *t*-test, or Mann-Whitney test were performed for comparisons of group means. A p value of less than 0.05 was considered statistically significant.

Conflict of interest

The authors state no conflict of interest.

Acknowledgement

We thank Wenjie Zheng for their excellent technical assistance. This research is supported by grants from the Public funding for healthcare, Ministry for Health, China (201202013).

References

Ahasan MM, Wakae K, Wang Z, Kitamura K, Liu G, Koura M, et al. APOBEC3A and 3C decrease human papillomavirus 16 pseudovirion infectivity. *Biochem Biophys Res Commun* 2015; 457:295-9.

Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, Howley PM. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J Virology* 1987; 61:962-71.

Burns MB, Temiz NA, Harris RS. Evidence for APOBEC3B mutagenesis in multiple human cancers. *Nat Genet* 2013; 45:977-83.

Choi JW, Ahn WS, Bae SM, Lee DB, Kim YW. Adenoviral p53 effects and cell-specific E7 protein-protein interactions of human cervical cancer cells. *Biosens Bioelectron* 2005; 20:2236-43.

Giannaki M, Kakourou T, Theodoridou M, Syriopoulou V, Kabouris M, Louizou E, et al. Human papillomavirus (HPV) genotyping of cutaneous warts in Greek children. *Pediatr Dermatol* 2013; 30:730-5.

Henderson S, Chakravarthy A, Su X, Boshoff C, Fenton TR. APOBEC-mediated cytosine deamination links PIK3CA helical domain mutations to human papillomavirus-driven tumor development. *Cell Rep* 2014; 7:1833-41.

Huo W, Di ZH, Xiao BH, Qi RQ, Weiland M, Gao XH. Clearance of genital warts in pregnant women by mild local hyperthermia: a pilot report. *Dermatol Ther* 2014; 27:109-12.

Huo W, Gao XH, Sun XP, Qi RQ, Hong Y, Mchepange UO, et al. Local hyperthermia at 44 degrees C for the treatment of plantar warts: a randomized, patient-blinded, placebo-controlled trial. *J Infect Dis* 2010; 201:1169-72.

Huo W, Li GH, Qi RQ, Zhang L, Yan XX, Chen HD, et al. Clinical and immunologic results of local hyperthermia at 44 degrees C for extensive genital warts in patients with diabetes mellitus. *Int J Hyperthermia* 2013; 29:17-20.

Koning FA, Newman EN, Kim EY, Kunstman KJ, Wolinsky SM, Malim MH. Defining APOBEC3 expression patterns in human tissues and hematopoietic cell subsets. *J Virology* 2009; 83:9474-85.

Kukimoto I, Mori S, Aoyama S, Wakae K, Muramatsu M, Kondo K. Hypermutation

in the E2 gene of human papillomavirus type 16 in cervical intraepithelial neoplasia. *J Med Virol* 2015; 87:1754-60.

Kukimoto I, Muramatsu M. Genetic variations of human papillomavirus type 16: implications for cervical carcinogenesis. *Jpn J Infect Dis* 2015; 68:169-75.

Li X, Gao XH, Jin L, Wang Y, Hong Y, McHepange UO, et al. Local hyperthermia could induce migrational maturation of Langerhans cells in condyloma acuminatum. *J Dermatol Sci* 2009; 54:121-3.

Ma Y, Huo W, Hong YX, Chen HD, Gao XH. Successful clearance of facial common warts by local hyperthermia: report of two cases. *Dermatol Ther* 2012; 25:386-8.

McBride AA. The papillomavirus E2 proteins. *Virology* 2013; 445:57-79.

Mehta HV, Jones PH, Weiss JP, Okeoma CM. IFN-alpha and lipopolysaccharide upregulate APOBEC3 mRNA through different signaling pathways. *J Immunol* 2012; 189:4088-103.

Moris A, Murray S, Cardinaud S. AID and APOBECs span the gap between innate and adaptive immunity. *Front Microbiol* 2014; 5:534.

Ostberg JR, Kabingu E, Repasky EA. Thermal regulation of dendritic cell activation and migration from skin explants. *Int J Hyperthermia* 2003; 19:520-33.

Ostrowska KM, Malkin A, Meade A, O'Leary J, Martin C, Spillane C, et al. Investigation of the influence of high-risk human papillomavirus on the biochemical composition of cervical cancer cells using vibrational spectroscopy. *Analyst* 2010; 135:3087-93.

Pattillo RA, Husa RO, Story MT, Ruckert AC, Shalaby MR, Mattingly RF. Tumor antigen and human chorionic gonadotropin in CaSki cells: a new epidermoid cervical cancer cell line. *Science* 1977; 196:1456-8.

Payne J, Nair MP, Ambrus JL, Chadha KC. Mild hyperthermia modulates biological activities of interferons. *Int J Hyperthermia* 2000; 16:492-507.

Refsland EW, Stenglein MD, Shindo K, Albin JS, Brown WL, Harris RS. Quantitative profiling of the full APOBEC3 mRNA repertoire in lymphocytes and tissues: implications for HIV-1 restriction. *Nucleic Acids Res* 2010; 38:4274-84.

Roberts SA, Lawrence MS, Klimczak LJ, Grimm SA, Fargo D, Stojanov P, et al. An APOBEC cytidine deaminase mutagenesis pattern is widespread in human cancers. *Nat Genet* 2013; 45:970-6.

Shterzer N, Heyman D, Shapiro B, Yaniv A, Jackman A, Serour F, et al. Human papillomavirus types detected in skin warts and cancer differ in their transforming properties but commonly counteract UVB induced protective responses in human keratinocytes. *Virology* 2014; 468:647-59.

Sunthamala N, Pang CL, Thierry F, Teissier S, Pientong C, Ekalaksananan T. Genome-wide analysis of high risk human papillomavirus E2 proteins in human primary keratinocytes. *Genom Data* 2014; 2:147-9.

Suspene R, Guetard D, Henry M, Sommer P, Wain-Hobson S, Vartanian JP. Extensive editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro and in vivo. *Proc Natl Acad Sci U S A* 2005; 102:8321-6.

Vartanian JP, Guetard D, Henry M, Wain-Hobson S. Evidence for editing of human papillomavirus DNA by APOBEC3 in benign and precancerous lesions. *Science* 2008; 320:230-3.

Vieira VC, Soares MA. The role of cytidine deaminases on innate immune responses against human viral infections. *Biomed Res Int* 2013; 2013:683095.

Wang X, Gao XH, Hong Y, Li X, Chen HD. Local hyperthermia decreases the expression of CCL-20 in condyloma acuminatum. *Viol J* 2010; 7:301.

Wang X, Gao XH, Li X, Hong Y, Qi R, Chen HD, et al. Local hyperthermia induces apoptosis of keratinocytes in both normal skin and condyloma acuminata via different pathways. *Apoptosis* 2009; 14:721-8.

Wang Z, Wakae K, Kitamura K, Aoyama S, Liu G, Koura M, et al. APOBEC3 deaminases induce hypermutation in human papillomavirus 16 DNA upon beta interferon stimulation. *J Virol* 2014; 88:1308-17.

Warren CJ, Xu T, Guo K, Griffin LM, Westrich JA, Lee D, et al. APOBEC3A functions as a restriction factor of human papillomavirus. *J Virology* 2015; 89:688-702.

Xue Y, Toh SY, He P, Lim T, Lim D, Pang CL, et al. HPV16-E2 induces prophase arrest and activates the cellular DNA damage response in vitro and in precursor lesions of cervical carcinoma. *Oncotarget* 2015; 6:34979-91.

Yee C, Krishnan-Hewlett I, Baker CC, Schlegel R, Howley PM. Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. *Am J Pathol* 1985; 119:361-6.

Zhu LL, Gao XH, Qi R, Hong Y, Li X, Wang X, et al. Local hyperthermia could induce antiviral activity by endogenous interferon-dependent pathway in condyloma acuminata. *Antiviral Res* 2010; 88:187-92.

Figure Legends

Figure 1. Representative examples of immunohistochemical staining of A3A and A3G on paraffin-embedded tissue sections. (a) A3A expression increased in CA as compared to normal skin. (b) A3G expression enhanced in CA compared with normal skin. Scale bar=40 μ m. Unpaired *t*-test was performed, yielding a P-value. ***p*<0.01.

Figure 2 APOBEC3A (A3A) and A3G expression increased after exposure to hyperthermia in a temperature-dependent manner. After exposure to specific hyperthermia, A3A and A3G mRNA expression were evaluated in CaSki, SiHa and C33A cells at 12 hours of hyperthermia treatment, and equivalent protein expression were assessed at 24 hours. (a,b) A3A and A3G mRNA levels increased at 42 °C and 45 °C in CaSki, SiHa and C33A cells. (c) A3A and A3G protein levels showed similar results.(d,e) A3A and A3G levels were measured between healthy controls and CA subjected to hyperthermia at 37 °C, 42 °C and 45 °C in vivo. Each symbol represents on individual donor. Horizontal bars represent means. One-way analysis of variance (ANOVA) and Mann-Whitney test were performed, yielding a P-value for all comparisons. **p*<0.05; ***p*<0.01 and ****p*<0.001.

Figure 3 3D-PCR analysis of the HPV16 E2 gene in cervical cell lines subjected to hyperthermia treatment. Hyperthermia caused increased A3A and A3G-induced mutations as compared with controls. (a) E2 3D-PCR assays were performed to

analyze the amplification products at a lower denaturation temperature in CaSki and SiHa cells. **(b)** The number of mutations for the edited HPV16E2 genomes was shown below the matrix in cervical cells treated with hyperthermia.

Figure 4 Regressing CA lesions receiving hyperthermia elevated mRNA expression of APOBEC3A (A3A) and A3G. (a,b) A3A and A3G mRNA expression in 6 CA patients treated with hyperthermia at 0min, 2 weeks, and 6 weeks, as measured by real-time PCR in vivo. **(c)** The total DNA samples from CA treated with hyperthermia at 0min, 2 weeks, and 6 weeks were analyzed in the E2 3D-PCR assay. **(d,e)** Summary of 3D-PCR amplicon sequences were shown as mutation matrices in the No. 4 (P4) presented with quick clinical response **(e)** and the other 5 patients **(d)**.

Figure 5 Inhibition of A3A or A3G decreased the proportion of A/T in E2 gene. The CaSki cells and SiHa cells were transfected with scrambled siRNA, two different A3A-specific siRNAs, and two different A3G-specific siRNAs, respectively. **(a,b)** Real-time PCR results and western blot analysis results were shown that both the A3A siRNAs downregulated the expression of A3A and the two A3G siRNAs inhibited the expression of A3G significantly. **(c)** The total DNA was extracted from CaSki cells and SiHa cells after transfection with siRNAs for 72 hours, and 3D-PCR results was shown that the A3A-siRNAs or A3G-siRNAs upregulated the denaturation temperature in HPV E2 sites. **(d)** Summary of 3D-PCR amplicon sequences were presented as mutation matrices.

Figure 6 Downregulation of A3A or A3G increased the cell viability of cervical cancer cells. CaSki , SiHa and C33A cells transiently downregulated A3A or A3G were cultured in 96-well plates. Cell viability was measured by MTS assay at day 1, 2, 3 and 4 as described under the Materials and Methods.

Figure 1

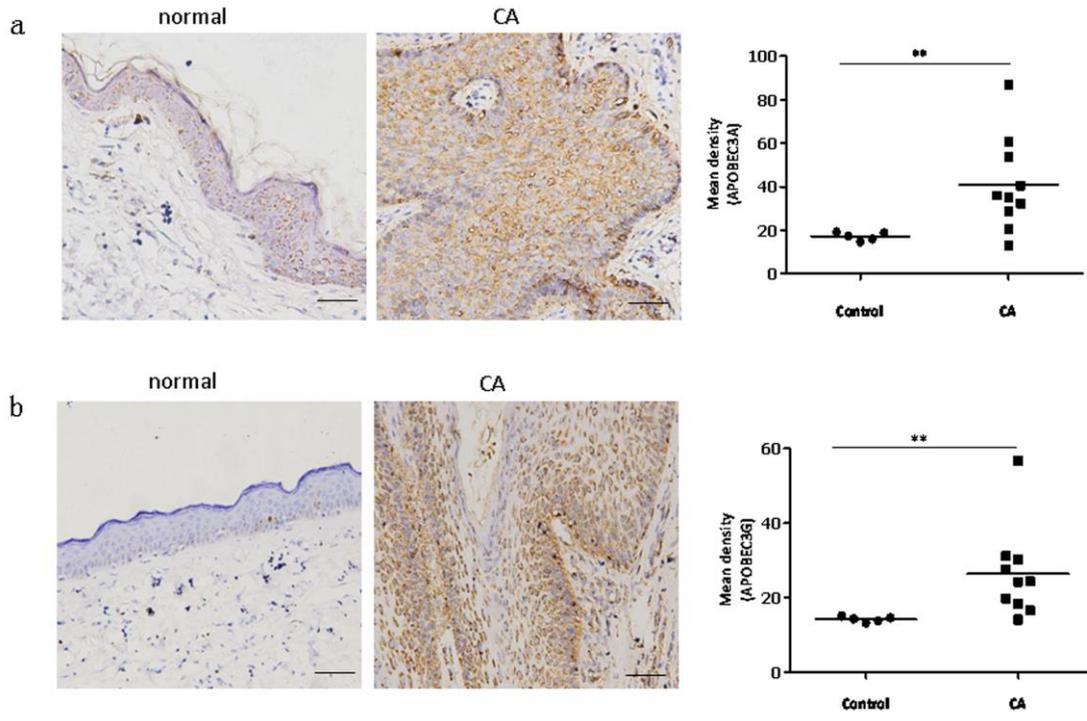


Figure 2

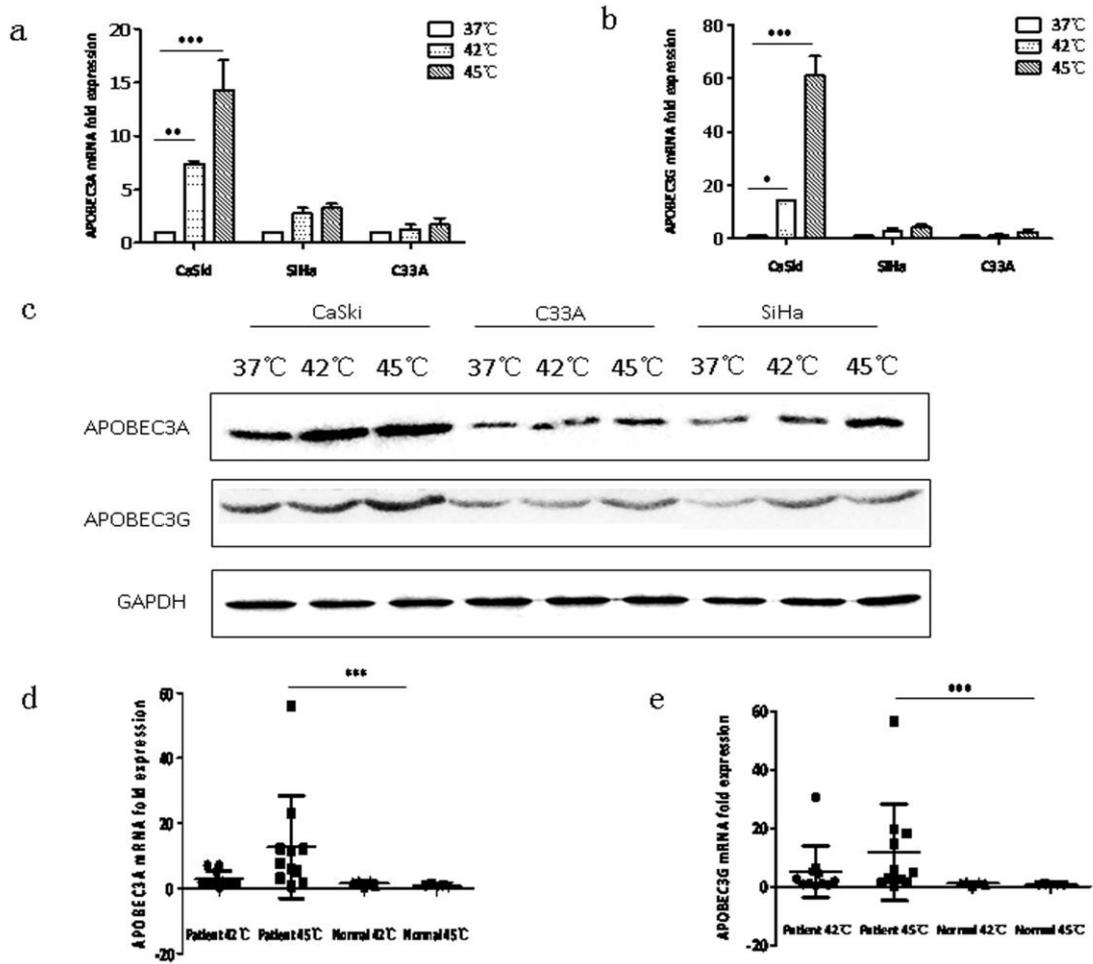


Figure 3

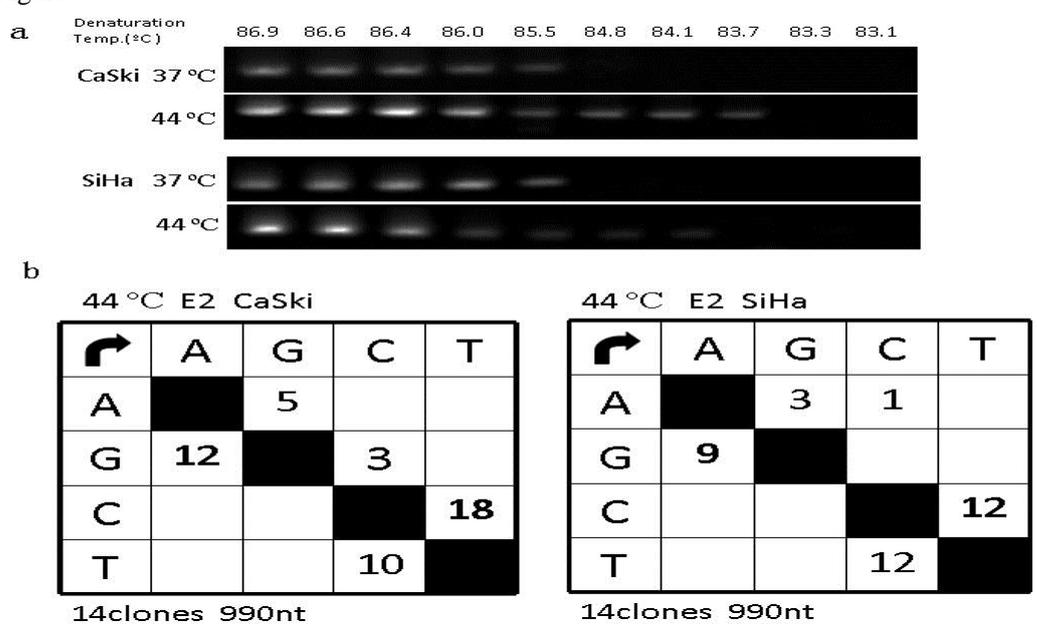


Figure 4

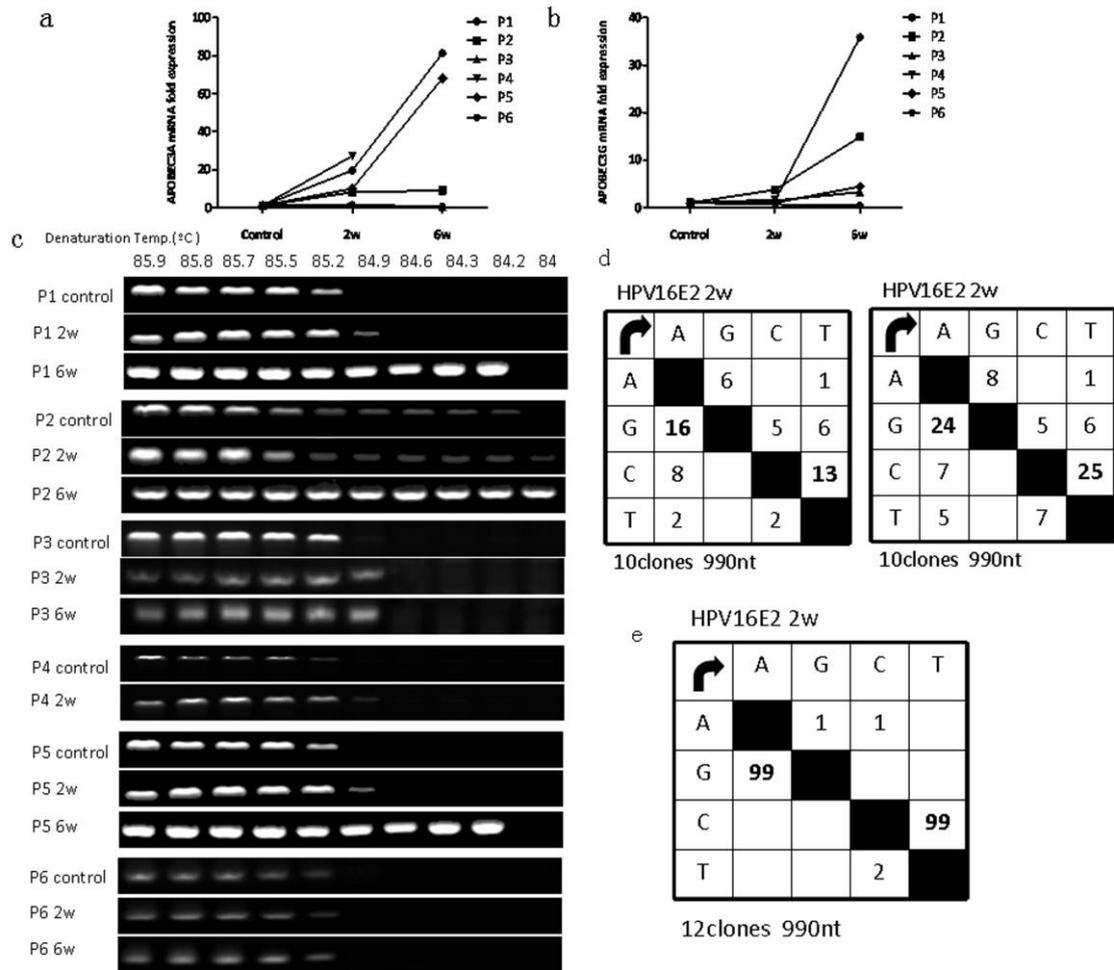


Figure 5

