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Local hyperthermia could induce antiviral activity by endogenous interferon-dependent pathway in condyloma acuminata

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ABSTRACT

Local hyperthermia has been successfully used in the treatment of viral warts by mechanisms that have largely remained unclear. Using an organotypic culture system, we found that hyperthermia at 42 °C and 45 °C could induce a significant increase in the transcriptional expression of interferon (IFN)-α, IFN-β and IFN-γ, in a temperature-dependent manner in condyloma acuminata (CA), but not in normal skin. Accordingly, local hyperthermia could enhance the expression of 2′-5′ oligoadenylate synthase and double-stranded RNA (dsRNA)-dependent protein kinase, two antiviral enzymes downstream of the IFN-dependant pathway. Hyperthermia led to an increase in IFN-α/β receptor transcripts, and an increase in the levels in phospho-Stat1 and phospho-Stat2 in CA, though it had no influence on the levels of Jak1, Tyk2, Stat1 and Stat2 transcriptional expression. Local hyperthermia was proved effective in treating human papillomavirus-infected skin. These results suggested that hyperthermia took effect partly by inducing the expression of local endogenous IFN and partly by subsequent IFN-induced antiviral activity via Jak–STATs signalling pathway in CA.

Local hyperthermia within the range of 42–49 °C has been successfully applied in the treatment of human papillomavirus (HPV)-infected skin lesions, with satisfactory tolerability and negligible side effects (El-Tonsy et al., 1999; Pfau et al., 1994; Stern and Levine, 1992). It has been postulated that local hyperthermia has direct antiviral effects, accelerates apoptosis of infected keratinocytes and inflammatory responses necessary for the eradication of the infected cells (Stern and Levine, 1992; Wang et al., 2009). Cell-mediated immune response is responsible for the eradication of HPV-infected keratinocytes and regression of the warty lesions (Hong et al., 1997; Li et al., 2009). Elevated temperatures could induce an increase in interferons (IFNs) production (Engervall et al., 1995; Huang et al., 1996; Park et al., 1990). Payne reported that mild hyperthermia could significantly enhance the antiviral activity of human IFN-α, -β and -γ and murine IFN-γ during antiviral assays (Payne et al., 2000).

IFNs are a family of biological response modifiers that exhibit mainly antiviral function. They consist of two different sub-types: type I IFNs (i.e., IFN-α/β) that are produced in virally infected cells, and type II IFN (i.e., IFN-γ) that is not virus inducible and is restricted to mitogen- or cytokine-activated lymphoid cells such as T lymphocytes and natural killer (NK) cells (Koromilas et al., 2001). IFN-α and β transduce their signals through the sequential activation of IFN-α/β receptor (IFNAR) (with two subunits, IFNAR1 and IFNAR2)-associated Janus tyrosine kinases Jak1 and Tyk2, leading to tyrosine phosphorylation and activation of Stat1 and Stat2. Activated Stat1/Stat2 heterodimers then translocate to the nucleus, where they associate with IFN regulatory factor IRF-9 to form an active complex (known as ISGF-3) on the IFN-stimulatory response element (ISRE) (Stark et al., 1998). This element is known to mediate the induction of a number of functionally important IFN-stimulated genes (ISGs), including double-stranded RNA (dsRNA)-dependent protein kinase R (PKR) and 2′-5′ oligoadenylate synthase (OAS) (Garcia et al., 2006; Randall and Goodbourn, 2008), to establish an antiviral response in target cells.

HPV infection in humans is usually through micro-trauma of the skin or mucosa, by way of which the virus can infect the germinal keratinocytes. Paralleled with programmed differentiation of keratinocytes, HPV assembles and matures, by temporal-spatial activation of different sets of genes (e.g., early (E) or late (L) genes). Here, by adopting a skin–organ culture procedure (Li et al., 2009; Ostberg et al., 2003; Wang et al., 2009), we tested whether controlled hyperthermia could induce endogenous IFNs and enhance IFN-induced antiviral activity through Jak–STATs signalling path-

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ways in condyloma acuminata (CA), with the aim of exploring the mechanisms by which hyperthermia treats HPV-infected skin.

1. Materials and methods

1.1. Patients and specimens

Seven patients (five males and two females) with clinically diagnosed CA were enrolled into the study. All enrolled patients had not undergone any prior anti-wart treatments. Patients with any documented systemic disease(s) were excluded from the study. Biopsies were obtained from genitals. Normal foreskin specimens ($n = 5$) from patients undergoing genital plastic surgery were obtained as normal controls upon obtaining informed consent.

This study was approved by the Ethics Committee of China Medical University.

1.2. Hyperthermia device and local hyperthermia protocol

As previously described (Gao et al., 2009; Li et al., 2009; Wang et al., 2009), we used a patented (Patent No. zl200720185403.3) local hyperthermia generator with an infrared-emitting source. The heat generated by the device acted on the skin surface without direct contact. The heated surface temperature was controlled and stabilised at the desired degrees with an accuracy of ±0.1 °C. A skin–organ culture protocol (Ostberg et al., 2003) was used in the study. Briefly, approximately 1 cm × 1 cm of sterile CA and normal skin specimens were trimmed to remove subcutaneous fat, and were cut into four equal portions. One of them was frozen and stored at −70 °C. Three pieces were separately placed in culture dishes, with dermal side down on the media (RPMI with 10% FBS, 2 mM glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 55 mM β-mercaptoethanol) and exposed to local heating at surface temperatures of 37 °C, 42 °C and 45 °C, respectively, for 30 min. Then they were fully submerged in culture media and incubated at 37 °C for 12 h with 5% CO_2. Finally, the specimens were embedded in Tissue-Tek® (Optimal Cutting Temperature (OCT) compound, Sakura, USA), frozen in liquid nitrogen and stored at −70 °C for subsequent handling.

1.3. DNA extraction and HPV typing

Total DNA was extracted from fresh skin specimens according to the instructions with the TIANamp Genomic DNA Kit (TIANGEN, DP304).

Polymerase chain reaction (PCR) amplification using HPV6 and 11 type-specific primers was performed as previously described (van den Brule et al., 1990). Briefly, the reaction system contained 2 μl DNA extraction, 2 μl 10× PCR buffer and 0.5 μl deoxynucleotide triphosphate (dNTP) mixture, forward and 2-μl reverse primer. Initial denaturation was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 60 s, then extended at 72 °C for 5 min. The amplification products were subjected to electrophoresis on 1.5% agarose gel and stained with ethidium bromide and visualised under ultraviolet (UV) light.

1.4. RNA isolation and fluorescent real-time (quantitative) PCR

Total RNA was extracted from specimens using RNAiso Plus (TaKaRa, Code: D9108A) and reverse-transcribed with PrimeScriptTMRT reagent Kit (TaKaRa, Code: DRR037S). The resulting complementary DNA (cDNA) was kept at −80 °C for further analyses. Real-time PCR analyses were performed on a Roter-Gene 3000 Detector System (Corbett Research, Australia). The primers used in this study were designed according to the GenBank sequence, as shown in Table 1. The reactions were carried out in a 20-μl reaction volume containing 10 μl SYBR® Premix Ex Taq^{TM}II (TaKaRa, Code: DRR081A), a 10-μM concentration of each forward and reverse primer, and 2 ng cDNA. The PCR conditions were as follows: Initial denaturation at 95 °C for 5 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 20 s and extension at 72 °C for 30 s. All assays were performed in triplicate.

Table 1
Primer for real-time PCR.

Name	Primer sequences (5′–3′)
β-actin	5′-TGGCACCCAGCACAATGAA-3′
	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′
IFN-α	5′-AAAGGCTGAAACCATCCCTGTC-3′
	5′-TCATCCCAAGCAGCAGATGAG-3′
IFN-β	5′-GCGACACTGTTCGTGTTGTCA-3′
	5′-CCAAGCAAGTTGTAGCTCATGGA-3′
IFN-γ	5′-CTTTAAAGATGACCAGAGCATCCAA-3′
	5′-GGCGACAGTTCAGCCATCAC-3′
IFNAR1	5′-GCGTACAAGCATCTGATGGAAATAA-3′
	5′-GATCACAGGCGTGTTTCCAGAC-3′
IFNAR2	5′-TTGCGAAATTTCCGGTCCA-3′
	5′-TTTGCACAGTTCTTAACCACCTTCA-3′
JAK1	5′-TGGCCCATTCATCAAGCTCA-3′
	5′-CACTTGTCAGCAGCCACACTCA-3′
TYK2	5′-ATGTGCCCATGGCTTGGAA-3′
	5′-CGGCCACACACATTACCATGA-3′
Stat1	5′-GGTACGAACTTCAGCAGCTTGACTC-3′
	5′-ACATCATTGGCAGCGTGCTC-3′
Stat2	5′-TTGCTACAGCGTCTGCTCCAC-3′
	5′-TGCTGCCAGTCTTGAGGATGA-3′
PKR	5′-TTGCGATACATGAGCCCAGAAC-3′
	5′-GCCATCCCGTAGGTCTGTGAA-3′
OAS1	5′-AGGTAGCTCCTACCCTGTGTGTGTG-3′
	5′-GAAGACAACCAGGTCAGCGTCA-3′

Relative quantification using the double standard curve method was performed on Roter-Gene 3000 Detector System using the Rotor-Gene software (Version 5.03).

1.5. Western blotting

Expressions of phospho-Stat1 (p-Stat1), Stat1 total, phospho-Stat2 (p-Stat2), Stat2 total, OAS1 and PKR proteins were evaluated by Western blot. Total extracts were prepared as described (Berglund et al., 2007). After measurement of protein concentration with a protein assay kit (Bio-Rad, Hercules, CA, USA), the protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride membrane (Millipore, CA, USA), and blocked with 0.1% Tween 20 and 5% skim milk overnight. The immunoblots were incubated with anti-OAS1, p-Tyr690 Stat1 (Santa Cruz, CA, USA), p-Tyr701 Stat2 (Abcam, CA, USA), PKR, Stat1and Stat2 (Bioworld, CA, USA) antibodies in TBS with 1% bovine serum albumin for 1 h. Anti-human β-actin antibody (ZSGB-Bio Corporation, Beijing, China) was used as a control. The membranes were washed 3 times with 0.1% Tween 20 in TBS and stained with horseradish peroxidase-conjugated secondary antibody. All immunoblots were detected by the Western blotting luminol reagent (Santa Cruz, CA, USA) according to the manufacturer's instructions.

1.6. Statistical analysis

Data were analysed with Statistical Package for Social Sciences (SPSS) 13.0 software. Quantitative results were expressed as mean ± SD. The differences between each group were analysed by one-way analysis of variance (ANOVA), and the corresponding significant results were further assessed by *post hoc* Multiple Comparisons (Least Significant Difference, LSD). *P*-values of less than 0.05 were considered statistically significant.

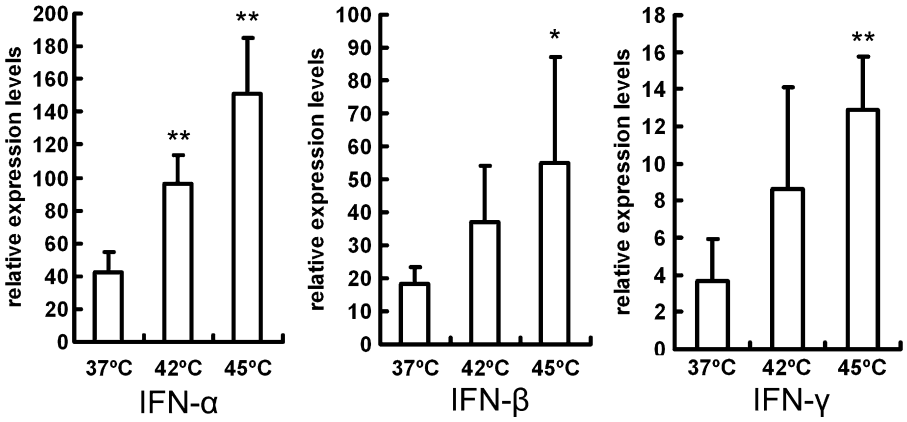

Fig. 1. The IFN-α, IFN-β and IFN-γ transcripts were all increased significantly in CA skin subjected to hyperthermia at 42 and 45 °C, as compared to that at 37 °C. *$p < 0.05$; **$p < 0.01$ vs. 37 °C.

2. Results

2.1. HPV typing in CA

A HPV-specific DNA segment was detected in all the seven CA specimens. Further sub-typings showed that three specimens were positive for HPV-6, one for HPV-11, and three for both HPV-6 and HPV-11.

2.2. Effects of hyperthermia on the induction of endogenous IFNs transcripts

As shown in Fig. 1, there was a significant increase in the production of IFN-α, -β and -γ transcripts in CA tissue that had received local hyperthermia. IFN-α, IFN-β and IFN-γ transcripts were increased significantly in CA tissue subjected to hyperthermia at 42 °C and 45 °C, as compared with that at 37 °C: the increase of IFN-α transcripts was 2–5- and 3–6-fold, that of IFN-β was 2–4- and 3–4-fold and that of IFN-γ was 2–5- and 4–14-fold, respectively (Fig. 1). There was no correlation between HPV types and the amount of IFN production in CA at any temperature ($p > 0.05$). The levels of IFN-α, -β and -γ transcripts were unaffected by the different hyperthermia temperatures applied to normal foreskin (data not shown).

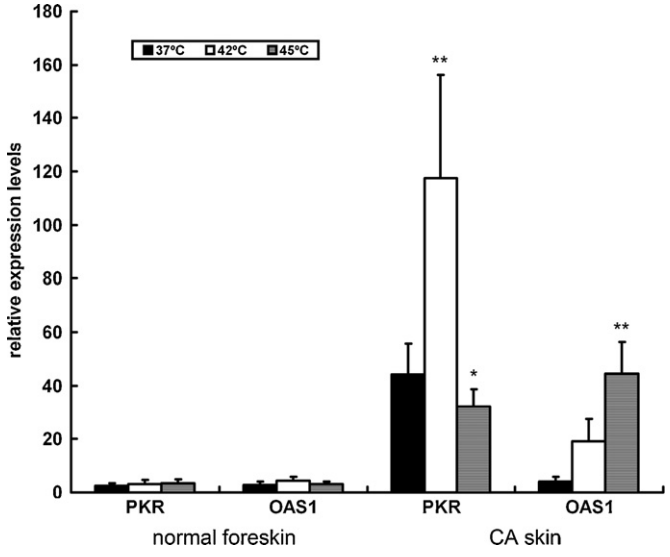

Fig. 2. The transcripts of both PKR and OAS1 remained stable when subjected to the different hyperthermia temperatures in normal foreskin (left). The PKR transcript was increased significantly at 42 °C while decreased at 45 °C, as compared to that of 37 °C; The OAS1 transcripts was increased significantly at 42 and 45 °C, as compared to that at 37 °C in CA skin (right). *$p < 0.05$; **$p < 0.01$ vs. 37 °C.

2.3. Effects of local hyperthermia on IFN-inducible enzymes

OAS and PKR are the major IFN-inducible enzymes that presumably are integrally linked to the antiviral activity of IFN (Garcia et al., 2006; Koscielniak et al., 1987); we thus examined the effect of hyperthermia on the induction of OAS1 and PKR in CA and normal skin. As shown in Fig. 2, the level of OAS1 transcripts was increased to about 5- and 11-fold when subjected to hyperthermia at 42 °C ($p < 0.05$) and 45 °C ($p < 0.001$), respectively, as compared with that at 37 °C; the corresponding proteins were increased to 1–2 folds at 42 °C ($p < 0.001$) and 45 °C ($p < 0.001$), respectively, as compared with that at 37 °C (Fig. 4). There was a 3-fold increase in PKR transcripts when the specimens were subjected to hyperthermia at 42 °C, but the level decreased to about 3/4 when subjected to hyperthermia at 45 °C, as compared with that at 37 °C (Fig. 2); The level of PKR protein, however, increased to about 1–2 folds when subjected to hyperthermia at 42 °C and 45 °C, as compared to that at 37 °C (Fig. 4). The expressions of both PKR and OAS1 remained stable when subjected to different hyperthermia temperatures in normal foreskin (Figs. 2 and 4).

2.4. Effect of local hyperthermia on IFN-α/β-receptor-associated kinases and signalling molecules in CA and normal foreskin

An increase in the number of IFNAR and/or IFNAR-associated kinases and signalling molecules in the tissue may be expected

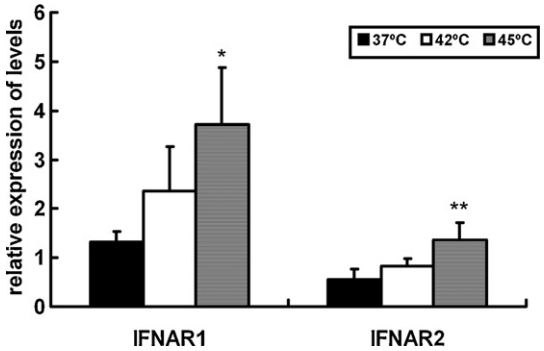

Fig. 3. Compared to 37 °C, the IFNAR1 and IFNAR2 transcripts were significantly elevated in specimens treated at 42 and 45 °C in a temperature-dependent manner in CA skin. *$p < 0.05$; **$p < 0.01$ vs. 37 °C.

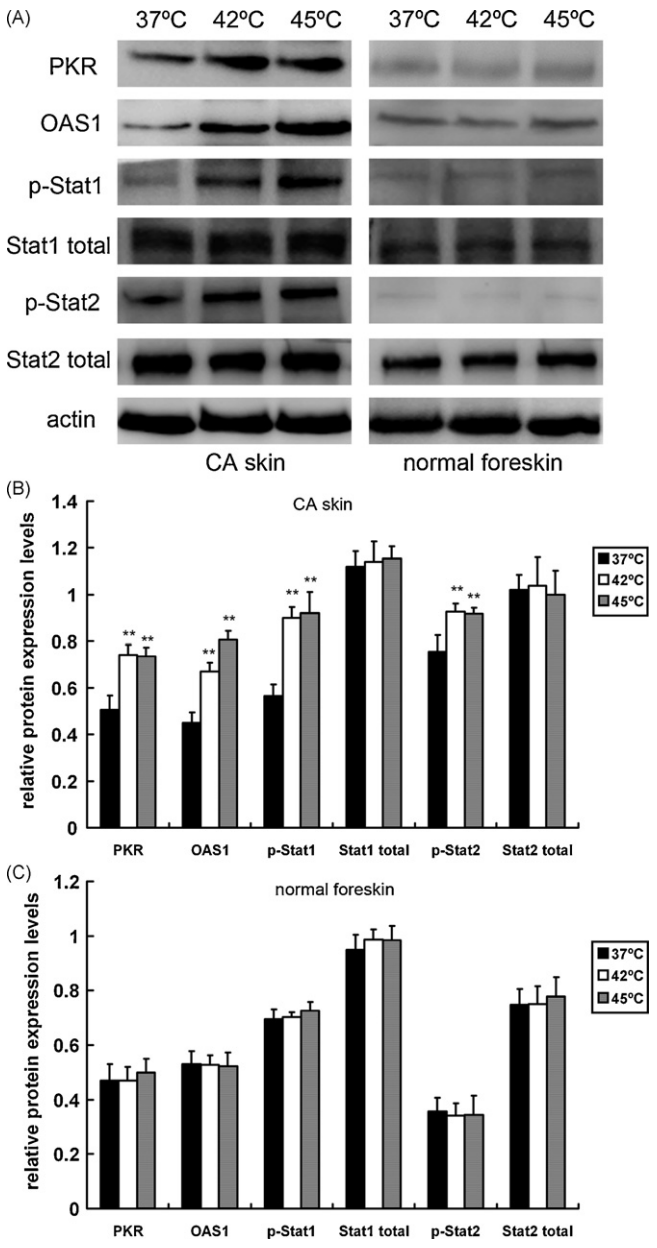

Fig. 4. The expressions of PKR, OAS1, p-Stat1, Stat1 total, p-Stat2, Stat2 total proteins in CA and normal foreskin were detected by Western blotting. The PKR, OAS1, p-Stat1, p-Stat2 protein were all increased significantly in CA skin at 42 and 45 °C, as compared to that at 37 °C. Both the Stat1 and Stat2 total protein remained stable at 42 and 45 °C in CA (A left). The assayed proteins were not influenced by hyperthermia temperatures applied in normal foreskin (A right). The bar charts show the relative protein expression levels of CA skin (B) and normal foreskin (C). The densities of corresponding bands were quantified by Image densitometry. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. 37 °C.

to give a greater induction of IFN-responsive enzymes. To investigate this possibility, we tested the sequence-specific transcripts of IFNAR (IFNAR1 and IFNAR2) and IFNAR-associated kinases and signalling molecules (Jak1, Tyk2, Stat1 and Stat2). Our results demonstrated that all tested mRNAs, except IFNAR1 and IFNAR2, remained stable in CA (data not shown), regardless of the hyperthermia temperatures applied. The level of IFNAR1 transcripts was increased to 2–3- and 3–5-folds when the specimens were subjected to hyperthermia at 42 °C ($p = 0.078$) and 45 °C ($p = 0.001$), respectively, as compared with that at 37 °C. The level of IFNAR2 transcripts was increased to 2–3- and 2–4-fold when subjected to hyperthermia at 42 °C ($p = 0.120$) and 45 °C ($p < 0.001$), respectively, as compared with that at 37 °C (Fig. 3).

The activation of the IRF-9/p-Stat1/p-Stat2 heterotrimer is the canonical mode of ISRE activation (Randall and Goodbourn, 2008). We detected the levels of p-Stat1 (Tyr690) and p-Stat2 (Tyr701) protein. As shown in Fig. 4, there was about 1–2-fold increase in p-Stat1 when the CA specimens were subjected to hyperthermia at 42 °C ($p < 0.001$) and 45 °C ($p < 0.001$), respectively, as compared with that at 37 °C. There was 1.5- and 1.8-fold increase in p-Stat2 when the CA specimens were subjected to hyperthermia at 42 °C ($p < 0.001$) and 45 °C ($p < 0.001$), as compared with that at 37 °C. The levels of IFNAR transcripts and p-Stat1 and p-Stat2 protein were not influenced by hyperthermia temperatures applied to normal skin (Fig. 4). Both the Stat1 and Stat2 total protein remained stable when subjected to hyperthermia at 42 °C and 45 °C, either in CA tissue or in normal foreskin (Fig. 4).

3. Discussion

Hyperthermia with temperatures ranging from 39 °C to 45 °C was effective in treating some tumours and HPV infections (El-Tonsy et al., 1999; Gao et al., 2009; Hildebrandt et al., 2005; Pfau et al., 1994; Stern and Levine, 1992). Our previous clinical trial showed that local hyperthermia temperatures at both 42 °C and 45 °C applied for 20–30 min were effective in the treatment of viral warts; the latter tended to have better cure rates (Gao et al., 2009; Huo et al., 2010). It was reported that hyperthermia between 39 °C and 42 °C promoted antiviral activities as a result of *de novo* syntheses of IFN-γ (Taylor et al., 1984). On the other hand, both *in vivo* and *in vitro* evidences show that HPV has developed a mechanism to evade the effects of IFN. HPV E7 protein is able to inhibit the antiviral activity of IFN-α and higher levels of E7 give better inhibition; this inhibition does not correlate with oncogenic potential but rather appears to be a general feature of all papillomaviruses (Barnard et al., 2000). HPV18 E6 has been shown to interact with the kinase Tyk2 in a fashion that limits its interaction with the IFN receptor and consequently impairs Jak-STAT activation (Li et al., 1999). In a separate study, we also found that hyperthermia could remarkably suppress the transcriptional expression of HPV-6/11 E6 and E7 (unpublished observation), a result orchestrating with the antiviral effect of hyperthermia by stimulating local IFN production.

We adopted an organotypic culture system to evaluate the effect of hyperthermia on the induction of endogenous IFNs and subsequent antiviral-related molecules in HPV-infected skin (Ostberg et al., 2003). We applied hyperthermia temperatures at 42 °C and 45 °C, using 37 °C as a control baseline condition. The present study showed that the expressions of endogenous IFN-α, -β and -γ transcripts were all increased when the CA specimens were subjected to hyperthermia temperature at 42 °C and 45 °C. The increase in IFNs transcripts was more prominent when the specimen was treated with higher temperature. There was no association of IFN induction with the HPV sub-types that infected the tissue (data not shown). The production of endogenous IFNs in normal foreskin was not affected by hyperthermia. It has been observed that the Epstein-Barr virus-transformed B-lymphoblastoid cell line could synthesise IFN-α and IFN-γ, while the non-transformed cell lines failed (Taylor et al., 1984). These results suggested that hyperthermia affected IFN production, in the condition where there was infective virus in the cells.

OAS1 and PKR are the two major enzymes involved in antiviral activities in the IFN-related pathway (Arany et al., 1999; Garcia-Milian et al., 1996; Gerein et al., 2004; Schoenfeld et al., 1995). We observed that hyperthermia could induce both transcriptional and translational production of OAS1 in specimens from CA while not from normal foreskin. Higher hyperthermia temperature tended

to be more efficient in OAS1 production. Similar findings have been observed in spontaneously transformed cells from human placental tissue in response to hyperthermia at 45 °C (Chousterman et al., 1987). Hyperthermia at 42 °C could up-regulate the transcriptional and translational expression of PKR. Hyperthermia at 45 °C, however, could up-regulate PKR protein expression, while it down-regulated its mRNA expression by about a quarter. Both PKR and OAS1 are IFN-stimulated genes (Garcia et al., 2006; Randall and Goodbourn, 2008). Hyperthermia itself may also affect the activation of transcription in a gene, though the mechanism of action has not been well characterised. It has been reported that hyperthermia-related gene transcription might be influenced by cell types, production of heat shock factors, the presence of heat shock response elements or even some unclassified heat-responsive sequences in a gene (Singh et al., 2002; Singh et al., 2000; Yan et al., 2002). Ishwar et al. reported that heating of a macrophage cell line at a sub-heat shock temperature (39.5 °C) had an effect comparable to the 37 °C control in the initial activation of tumour necrosis factor (TNF) transcription response; however, the duration of transcriptional activation was markedly reduced in the former (Singh et al., 2000). There is a potential heat shock response element in the promoter region of PKR (Yan et al., 2002). We speculated that, in addition to the IFN pathway, hyperthermia directly affected the transcription of PKR. Though the precise regulatory action is not clear, hyperthermia temperature at 45 °C might suppress its transcription activation to such an extent that it attenuated the enhancing role of IFNs. There has been no report on the direct response of OAS1 to hyperthermia treatment. Dubois et al. reported that IFN-induced PKR protein was strongly decreased during a heat shock, and 6 h at 37 °C after the heat shock it was increased, possibly due to *de novo* synthesis. He also noted that enhanced translation of PKR could continue up to 20 h after treatment with IFN (Dubois et al., 1989). In the present study, we did not assay the levels of PKR in samples immediately after hyperthermia treatment. However, the samples were cultured for another 12 h at 37 °C after hyperthermia treatment, a period of time long enough for IFN-stimulated translation of PKR. Though there was a moderate decrease of PKR transcripts, enhanced translation of PKR by IFN over a sufficient period of time might result in a net increase in PKR level. Hyperthermia-induced up-regulated expression of OAS1 and PKR was favourable for suppression of HPV *de novo*. To determine if hyperthermia could influence the key components in the IFN pathway, we examined the transcriptional expression of IFNAR and IFN-α/β-receptor-associated kinases and signalling molecules. There was a significantly increased transcriptional expression of IFNAR1 and IFNAR2 in CA specimens exposed to hyperthermia, while there was no change in that of normal skin under the same condition. Moreover, transcriptional expressions of Jak1, Tyk2, Stat1 and Stat2 were not influenced by hyperthermia treatment in both CA and normal skin. However, the levels of p-Stat1 and p-Stat2 protein were both significantly increased in specimens treated at hyperthermia temperatures of 42 °C and 45 °C in CA, and this increase was not due to an increase in total Stat1/2 proteins. The increased expressions of p-Stat1 and p-Stat2 also favour the activity of their downstream antiviral enzymes, such as OAS1 and PKR. In all, these findings demonstrated that the enhancement of IFN-induced antiviral activity by hyperthermia occurred both at the membrane receptor level and at the later step, in the transducing signal generated after IFN/receptor interaction.

This study in part explained the clinical effectiveness of hyperthermia in the treatment of HPV-infected skin, which had been practiced for years. Furthermore, by understanding the mechanism involved in hyperthermia-induced up-regulation of antiviral activity, more efficient ways might be designed to deal with HPV infection of the skin.

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The authors declare that there are no conflicts of interest.

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