Proteomic and bioinformatic analysis of condyloma acuminata: mild hyperthermia treatment reveals compromised HPV infectivity of keratinocytes via regulation of metabolism, differentiation and anti-viral responses

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ABSTRACT
Background: Hyperthermia has proved successful in treating cutaneous human papillomavirus infectious diseases such as plantar wart and condyloma acuminata (CA). Moreover, this treatment provides improved therapeutic efficacy in these conditions as compared with conventional therapies.

Objectives: To investigate the global proteome changes in CA in response to hyperthermia and achieve a better understanding of the mechanisms of hyperthermia therapy against HPV-infectious diseases.

Methods: CA tissue was obtained from patients undergoing pathological examinations. Diagnosis was verified as based on results of both HE staining and HPV-DNA PCR assay. Hyperthermia was achieved with a 44°C water bath. Differentially expressed proteins (DEPs) were identified by iTRAQ labeling, SCX chromatography and LC-MS/MS assay. Validation of proteomic results was performed using real-time qPCR and western blot, while bioinformatic analysis of DEPs was accomplished by R 3.4.1, STRING and Cytoscape softwares.

Results: In response to hyperthermia, a total of 102 DEPs were identified with 37 being upregulated and 65 downregulated. Among these DEPs, hyperthermia induced proteins involved with anti-viral processes such as OAS1, MX1, BANF1, CANX and AP1S1, whereas it inhibited proteins that participated in cellular metabolism, such as GALT, H6PD, EXOSC4 and EXOSC6; protein translation, such as RPS4Y1; as well as keratinocyte differentiation, such as KRT5, KRT27, KRT75, KRT76 and H2AFY2.

Conclusions: Hyperthermia inhibited enzymes and molecules responsible for metabolism modulation and keratinocyte differentiation in CA tissue, whereas it promoted factors involved in anti-viral responses. Such effects may, in part, contribute to the efficacy of local hyperthermia therapy against HPV infection.

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Introduction
Condyloma acuminatum (CA) is a common, sexually-transmitted disease observed worldwide that develops from human papillomavirus (HPV) infection. Among all HPV subtypes, CA is primarily the result of either a HPV 6/11 (low-risk types) or HPV 16/18 (high-risk types) infection [1]. CA patients are typically treated with physical therapies such as CO2 laser ablation and cryotherapy, however, recurrence is oftentimes observed due to failures in eliminating invisible HPV-infected loci [2]. Although 5-aminolaevulinic acid photodynamic therapy (ALA-PDT) is effective in treating latent HPV infection and reducing recurrence of CA [2], the relatively high medical expenses and pain associated with this therapy limits its application, especially in patients with massive lesions. Therefore, there exists a clear need for the development of more effective and economic therapeutic methods.

Local hyperthermia has been widely used as an adjunctive therapy in the treatment of various cancers including cervical [3], melanoma [4], breast [5] and bladder [6] cancers. Within our clinic, we have also applied local hyperthermia as a means for treatment of several cutaneous human papillomavirus (HPV) infectious diseases such as plantar wart [7] and CA [8]. This has proved a valuable approach as indicated by improved therapeutic efficacies and reductions in recurrence rates as compared to conventional therapies [9]. Preliminary results from our, as well as other, laboratories have revealed that the therapeutic effects of local hyperthermia in treating cancers and HPV-infectious diseases were, in part, due to its...
immune-stimulating actions resulting in enhanced antigen presentation [10] and/or the induction of immune-active molecules [11]. However, much of the underlying mechanisms of local hyperthermia therapy, especially in treatment against HPV infection, remains unknown and requires further investigation.

Isobaric tags for relative and absolute quantitation (iTRAQ) is a quantitative proteomics technology which uses covalently-bonded isotope labeled molecules to detect the simultaneous expressions of global proteins in up to 8 samples [12,13]. As a powerful proteomic detection tool, iTRAQ technology possesses a high degree of quantification accuracy and reproducibility [14,15] and thus is extensively implemented in proteomic comparisons among different samples or treatments. Therefore, as an approach to achieve an overview of protein alterations during local hyperthermia therapy, we applied iTRAQ technology to evaluate protein expression changes at the proteomic level as resulting from ex vivo hyperthermia treatment in CA tissue.

To our knowledge, this is the first proteomic detection of differentially expressed proteins in CA tissues in response to hyperthermia. The resultant bioinformatics analyses of DEPs offer considerable instructive information about the underlying mechanisms for the therapeutic effects of local hyperthermia. Such insights not only provide the foundation for the design of subsequent studies, but will enable an improved efficacy in the use of local hyperthermia therapy.

Materials and methods

Cell culture

HaCaT and CaSkI cells were purchased from GeneChem (Shanghai, China) and cultured in a cell incubator at 37 °C, 5% CO2, in Dulbecco’s Modified Eagle Medium (HaCaT cells; Biological Industries, Israel) and RPMI 1640 medium (CaSkI cells, Biological Industries, Israel) containing 10% fetal bovine serum and 1% penicillin and streptomycin (Biological Industries, Israel). Cells were subcultured using 0.25% trypsin with 0.02% EDTA (Biological Industries, Israel) and seeded for further experiments when cells achieved a confluence of 70–80%.

Tissue and pathological diagnosis

Fresh CA tissue was obtained from patients that underwent pathological examinations at The First Hospital of China Medical University. All CA tissue samples were dissected into quadrants and assigned to one of the following four conditions: HE staining (1), DNA extraction (1) and hyperthermia treatment (2). For HE staining and pathological diagnosis, CA tissues were first fixed in 4% paraformaldehyde for over 24 h at room temperature followed by sequential dehydration through a series of ethanol gradients and then embedded in paraffin wax. Embedded tissues were sectioned (6 μm thickness) with a microtome and then stained with hematoxylin and eosin (Solarbio, China). After staining, the slices were covered with a coverslip, mounted with one drop of neutral resin (Solarbio, China) and then forwarded to a pathologist for microscopy inspection.

DNA extraction and PCR assay

Cell cultures were digested with use of 0.25% trypsin (Biological Industries, Israel), washed 3 times with PBS and collected following centrifugation (800 g for 5 min). CA tissue was ground into a fine powder in liquid nitrogen. DNA was then extracted from each sample with the use of Dnasey Blood & Tissue Kit (Qiagen, Germany) following the manufacturers’ instructions. Extracted DNA samples were then subjected to PCR assay with use of a Veriti 96 Well Thermal Cycler (Applied Biosystems, USA). The primers used for HPV DNA amplification were as follows:

FAP59/64 sense - 5'-TAACWGTTGGICAYCCWTATT-3'
FAP59/64 antisense - 5'-CCWATATCWHCATITCICCATC-3'
GP5+/6+ sense - 5'-TTTTGACTGTTGATAGATCTAC-3'
GP5+/6+ antisense - 5'-GAAAAATAACTGTAACATCATATC-3'

Each PCR reaction mixture contained 12.5 μl of GoTaq Colorless Master Mix (Promega, USA), 2 μl of DNA sample, 1 μl of each primer and 8.5 μl nuclease-free water to achieve a final volume of 25 μl. The PCR reaction condition was set to 94 °C for 10 min, followed by 45 cycles of 94 °C for 1.5 min, 50 °C for 1.5 min and 72 °C for 1.5 min. The reaction was then terminated at 72 °C for 10 min.

After PCR amplification, all samples were subjected to an agarose gel electrophoresis assay. The agarose gel was prepared with the use of 0.5 g agarose gel powder (Sigma Aldrich, USA), 50 ml of 1% TBE buffer (Solarbio, China) and 5 μl of type I nucleic acid dye (Solarbio, China). Samples (10 μl) were loaded onto the agarose gel and separated at 100 V for 30 min at room temperature. After electrophoresis, the gel was exposed under UV light in a MF-ChemiBIS 2.0 Bio-Imaging Systems (DNR, Israel).

Hyperthermia treatment

Two of the dissected CA tissue samples from each patient were separately immersed in RPMI 1640 medium within 60 mm Petri dishes and assigned either to the hyperthermia or control group. All hyperthermia group dishes were carefully wrapped with paraffin and subjected to a 44 ± 1 °C water bath (Thermo Fisher Scientific, USA) for 30 min, whereas control group dishes were treated likewise in a 37 °C water bath (Thermo Fisher Scientific, USA). After hyperthermia treatment, all the dishes were returned to the 37 °C incubator for a 6 h recovery before protein extraction or a 2 h recovery before mRNA extraction.

Protein extraction and isobaric labeling

CA tissue samples from each group were ground into a fine powder in liquid nitrogen, and then mixed with 5 times volume of TCA/acetone (1:9) by vortexing, maintained at -20 °C for over 4 h, washed 3 times with pre-cooled acetone and desiccated in a fume hood. Dried tissue powder (30 mg) was suspended in 30 times volume of SDT buffer (4% SDS, 100 mM Tris-HCl, 1 mM DTT, pH7.6). The suspension was then vortexed, sonicated and heated in a boiling bath for 5 min.
centrifugation at 14,000 g for 40 min, the supernatant was collected, filtered with use of a 0.22 μm microfiltration membrane (PALL, USA) and quantified using the BCA method (P0012, Beyotime, China). FASP digestion was performed using 4 μg Trypsin per 200 μg protein and peptide quantification was performed according to Wisniewski, et al. [16]. Each peptide sample (100 μg) was labeled with use of the iTRAQ Reagent – 8plex Multiplex Kit (AB SCIEX) following protocols supplied. Six CA samples were labeled with iTRAQ tags 113, 114 or 115 for control patients 1, 2 and 3, and 116, 117 or 118 for hyperthermia patients 1, 2 and 3, respectively. The 6 labeled samples were then pooled for further experiments.

**SCX chromatography**

SCX chromatography was performed with the help of Shanghai Applied Protein Technology. Briefly, labeled peptides were fractionated by SCX chromatography using the AKTA Purifier system (GE Healthcare). The dried peptide mixture was reconstituted, acidified with buffer A (10 mM KH2PO4 in 25% of ACN, pH 3.0) and loaded onto a PolySULFOETHYL 4.6 x 100 mm column (5 μm, 200 Å, PolyLC Inc, Maryland, USA). The peptides were eluted at a flow rate of 1 ml/min with a gradient of 0–8% buffer B (500 mM KCl, 10 mM KH2PO4 in 25% of ACN, pH 3.0) for 22 min, 8–52% buffer B during min 22–47, 52–100% buffer B during min 47–50, 100% buffer B during min 50–58, and buffer B was then reset to 0% after 58 min. The elution was monitored by absorbance at 214 nm and fractions were collected every 1 min. A total number of 10 fractions were collected, desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 ml, Sigma) and concentrated by vacuum centrifugation.

**LC-MS/MS and protein identification**

Mass spectrometry was performed with the help of Shanghai Applied Protein Technology. In brief, fractionated samples were separated with use of a nano HPLC system Easy nLC (Thermo Fisher Scientific, USA), and then subjected to a Q Exactive mass spectrometer (Thermo Fisher Scientific, USA) that was operated in a positive ion mode. MS parameters were set as follows:

- Survey scan range – 300–1800 m/z for HCD fragmentation
- Automatic gain control (AGC) target – 3e6
- Maximum inject time – 10 ms
- Dynamic exclusion duration – 40.0 s
- Survey scans resolution – 70,000 at 200 m/z
- Hcd spectra resolution – 17,500 at 200 m/z
- Isolation width – 2 m/z
- Normalized collision energy – 30 eV
- Underfill ration – 0.1%

For protein identification, Q Exactive raw data were processed with the use of Proteome Discoverer 1.4 software (Thermo Fisher Scientific, USA) and searched against the UniProt HomoSapiens protein database downloaded from http://www.uniprot.org (Uniprot_HomoSapiens_154578_20160822.fasta) using Mascot 2.2 (Matrix Science, UK).Search parameters were set as follows:

- Digestion enzyme – Trypsin
- Max missed cleavages – 2
- Fixed modifications – Carbamidomethyl (C), itraq 8plex (N-term and K)
- Variable modifications – Oxidation (M), itraq 8plex (Y)
- Peptide mass tolerance – ±20 ppm
- Fragment mass tolerance – 0.1 Da
- Protein mass – Unrestricted
- Database pattern – Decoy
- Peptide FDR – ≤0.01
- Protein quantification – Protein ratios were calculated as the median of only unique peptides of the protein
- Experimental bias – Normalizes all peptide ratios by the median protein ratio.

Only proteins that were identified in all 6 samples were selected. The expression changes of identified proteins were calculated by dividing the iTRAQ reporter intensities of hyperthermia groups by that of control groups. Differentially Expressed Proteins (DEPs) were recognized when they met a criteria of 1.2-fold change (>1.2 or <0.833) and an associated paired t-test p values < .05.

**Bioinformatics analysis**

Annotation of identified proteins and gene ID conversion were performed using the org.Hs.eg.db package (Rstudio based on R3.4.1, Boston, MA). Differentially Expressed Proteins (DEPs) were categorized by k-means clustering and hierarchical clustering using the cluster package (Rstudio based on R3.4.1, Boston, MA). The optimal cluster number was determined using gap statistics and clustering results were visualized with the use of the heatmap package (Rstudio based on R3.4.1, Boston, MA). For functional analyses of DEPs, Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed with use of the clusterProfiler package (Rstudio based on R3.4.1, Boston, MA) according to Yu et al. [17]. The enrichment score was calculated from the p values of enrichment analysis (−log10(p values) × 10). The protein-protein interaction (PPI) network was constructed with use of the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, http://www.string-db.org/). Interaction modules and hub-proteins were analyzed with use of MCODE and CenTiScaPe plugins in Cytoscape 3.5.1 with default parameters. Modules with a MCODE score greater than 2 were selected for further analysis. Proteins with a centrality score greater than the threshold value of 2.7755 were considered as hub-proteins.

**Real-time quantitative PCR assay**

Three fresh CA tissue samples were collected, bisected, assigned to either the hyperthermia or control group and...
subjected to the experimental conditions as described above. The mRNA of each sample was extracted with use of the miRNeasy Mini Kit (Qiagen, Germany) according to the protocols supplied and quantified with use of a ND1000 nanom-eter. The mRNA (1 μg) was then utilized as templates for synthesis of complementary DNA (cDNA) with use of the GoScript™ Reverse Transcription System (Promega, USA) following the manufacturers’ instructions.

Primer sequences for OA51 (sense 5'-ACTACCAGCAACTT GCATCTA-3', antisense 5'-CTCCACACCAAGTGGTCC-3'), MX1 (sense 5'-CCTCTGTTCCACCTGAAAGA-3', antisense 5'-CTCCAG CAGATCCCTGAAATATG-3'), AP151 (sense 5'-TGGCGAGCTGG ACATCAT-3', antisense 5'-CATCCCTCCTTGCACTAGG-3'), BANF1 (sense 5'-GCTGGATGTTGGAAGTCCT-3', antisense 5'-ACAAGTGTCTTTCAGCCATCC-3'), CANX (sense 5'-CTGGTTATC CTCTTCTGGTTC-3'), antisense 5'-CTTCTCTCTCCCTCTCCAT C-3'), EXOSC4 (sense 5'-AAGCAGCAGCAGCCACA-3', antisense 5'-CACAAAGTCTCCTGTGGGTAGTCC-3'), GALT (sense 5'- CATGCTGGTCCAGCAATGC-3', antisense 5'-GTTGCTGTCTCC CTGTCCCT-3'), KRT5 (sense 5'-AGATCGCCACTTACGCGA-3', antisense 5'-GCACATCCAGAAGAACACTG-3'), EXOSC6 (sense 5'-AGATGCAGCAGCTGGTG-3', antisense 5'-CTGATT CAGCAGCAGCTGG-3'), KRT76 (sense 5'-AAGGCTAAGGATG ACCTGGG-3', antisense 5'-CACACGCGACTCTCATCT-3') and GAPDH (sense 5'-AAGGCGACAGGAAGAGAAGA-3', antisense 5'-GTCATACGGCAACTGAGG-3') were designed using PrimerPremier 6.0 software (Palo Alto, CA) and BLAST against the NCBI database.

The mRNA expression levels were quantified by the 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) with use of RT2 SYBR Green qPCR Mastermix (Promega, USA) following the protocol supplied. GAPDH was used as an endogenous loading control.

Protein extraction and Western blotting

Four fresh CA samples were collected, bisected and subjected to similar hyperthermia treatment as indicated above, and then recovered for 6 h at 37 °C, washed briefly with ice-cold PBS and grounded into a fine powder in liquid nitrogen. Each tissue powder was lysed using 300 μl RIPA buffer (Beyotime, China) premixed with PMSF (Beyotime, China), protease inhibitor cocktail (Roche, Switzerland) and phosphatase inhibitor (Roche, Switzerland) according to the manufacturer's protocols. After centrifugation at 4 °C for 15,000 g for 10 min, the supernatants were collected and quantified with use of the BCA protein assay kit (Beyotime, China).

For western blot assay, 20 μg of each sample protein was loaded with SDS buffer (Beyotime, China) and denatured at 95 °C for 10 min in a metal bath. Primary antibodies used in western blotting were rabbit anti-human GALT (ab178406, Abcam, UK), keratin-5 (#25807, Cell Signaling Technology, USA), Calnexin (#2679, Cell Signaling Technology, USA), MX1 (#37849, Cell Signaling Technology, USA), GAPDH (ab181602, Abcam, UK) monoclonal antibodies and mouse anti-human keratin-76 monoclonal antibody (ab16111, Abcam, UK). The secondary antibodies were HRP-conjugated goat anti-rabbit and anti-mouse IgG polyclonal antibody (ZB-2301 and ZB-2305, ZSGB-BIO, China). Antibodies were all diluted according to instructions applied.

Statistics and figures

Paired t-tests were utilized for analyzing proteomic, real-time qPCR and western blotting results of CA tissues. A p value <.05 was required for results to be considered statistically significant. Results were expressed as the mean ± SEM. All statistical calculations were performed with use of R 3.4.1 software and figures were drawn with use of the ggplot2 package in R software (Rstudio based on R3.4.1, Boston, MA).

Results

Diagnosis of CA as determined by HE staining and HPV identification

A biopsy and HE staining were performed on samples dissected from tissues of the 3 patients and were then evaluated by a dermatologic pathologist. Pathological examination revealed characteristic changes such as papillomatosis, koilocytes in the stratum spinosum, parakeratosis in stratum corneum, enlargement of dermal papillae and expansion of dermal capillaries (Figure 1(A)). A subsequent HPV-DNA PCR assay using FAP59/64 primer pairs (480 bp) and GP 5+/6+ primer pairs (150 bp) verified the existence of HPV DNA copies in tissue samples of the patients (Figure 1(B)). HaCaT cells were used as negative controls, whereas CaSki cells were used as positive controls of HPV-DNA copies. Taken together, a diagnosis of CA was made and the remaining samples of the 3 patients were then subjected to further experiments.

Quantitative proteomic analyses identified differentially expressed proteins in CA tissue subjected to hyperthermia treatment

To examine potential protein alterations resulting from local hyperthermia therapy, CA tissues in the presence or absence of ex-vivo 44 °C hyperthermia treatment from 3 patients were subjected to an iTRAQ-based quantitative proteomic analysis following procedures illustrated in Figure 2(A). Based upon the criteria described above, a total of 20,208 unique peptides that matched to 4128 non-redundant proteins were identified. As screened by a 1.2-fold cut-off criterion (treatment group/control group >1.2 or <0.833) together with a paired t-test p values <.05, 102 proteins were ultimately identified as differentially expressed proteins (DEPs, Table S1), among which 37 were up-regulated and 65 down-regulated (Figure 2(B)). The top 10 up and down-regulated proteins are shown in Table 1.

Validation of gene expressions on mRNA levels using RT-qPCR assay

Protein alterations in CA tissue induced by hyperthermia may likely be due to increased transcriptions. Therefore, a
real-time qPCR assay was utilized to assess mRNA changes of 10 selected proteins identified from the iTRAQ assay (OAS1, MX1, AP1S1, BANF1, CANX, EXOSC4, GALT, KRT5, EXOSC6 and KRT76). For this procedure, 3 new CA tissue samples were collected, bisected and treated as described above. The RNA samples were extracted after a 2-h recovery period in 37°C. The qPCR results revealed that a significant mRNA up-regulation was observed for MX1, AP1S1, BANF1 and CANX (Figure 3(B,C,D,E)), while EXOSC4, GALT, KRT5 and EXOSC6 were all downregulated (Figure 3(F,G,H,I)). Although mRNA of OAS1 was up-regulated (Figure 3(A)) and KRT76 downregulated (Figure 3(J)), these changes failed to achieve a statistically significant difference due to variations of values within samples. The results obtained from qPCR were closely related with those obtained with the iTRAQ assay, which validated the results of protein alterations observed in CA tissues subjected to hyperthermia.

Validation of gene expressions on protein levels using Western blot assay

For direct protein expression validation, five major target genes identified from the iTRAQ assay (CANX, MX1, KRT5, KRT76 and GALT) were detected by western blotting using specific monoclonal antibodies, and GAPDH was used as a loading control. Similar to qPCR assay, the western blot assay showed that the protein levels of KRT5, KRT76 and GALT were majorly down regulated in CA samples in response to ex-vivo hyperthermia, whereas MX1 was up-regulated. CANX, however, did not show obvious alteration in protein expression (Figure 3(K)). Additionally, a grayscale analysis of western blot result was performed with use of Adobe Photoshop 7.0 software. A paired t-test of the grayscale values indicated that among all four CA samples the protein alterations of MX1 and KRT76 were statistically significant whereas alterations of CANX, MX1 and KRT5 were not (Figure 3(L)). Further considering the grayscale value of each individual sample, it can be inferred that the statistical insignificance of MX1 and KRT5 were due to individual variations. In conclusion, these results demonstrated that the protein levels of many target genes were altered in CA tissues during hyperthermia treatment. Moreover, it should be noted that individual protein...
expression variations existed within different samples during ex-vivo hyperthermia treatment. Such variation may, in part, explain the differences observed in therapeutic responses of patients receiving local hyperthermia treatment against HPV-infectious diseases in clinics.

### Enrichment analysis of DEPs revealed altered functions in relation to metabolism, differentiation and anti-viral activity in CA tissue subjected to hyperthermia

The 102 DEPs identified were first subjected to GO enrichment analysis with the use of the clusterProfiler package in R 3.4.1 software. The enriched terms of biological process (BP), molecular function (MF) and cellular component (CC) were sorted by gene count and the top ten terms of the three GO categories with a p value < .05 are summarized in Figure 4(A). The results revealed that the DEPs obtained principally participated in metabolic and catabolic processes as well as defense activities against viruses and other organisms (BP). These effects were exerted through influencing molecular reactions involving phosphor- and acyl-groups and other enzymes (MF) within the nucleus, mitochondria and cell skeleton (CC). A KEGG enrichment analysis was then performed on DEPs using the same R package. The results were sorted as described above and the top ten KEGG terms are presented in Figure 4(B). Similarly, the DEPs participated in defense activity related pathways, such as human papilloma-virus infection, influenza A, phagosome and protein processing in endoplasmic reticulum; metabolism and cell survival-related pathways, such as pentose prophase, PI3K-Akt and Hippo signaling pathways; and cell skeleton and structure related pathways, such as tight and adherens junctions.

### Protein-protein interaction analysis for the identification of interaction modules and hub proteins of DEPs in CA tissue subjected to hyperthermia

To explore possible interactions in the relationships of the 102 DEPs, the STRING online database was utilized to calculate protein-protein interaction (PPI) scores. After establishing a threshold interaction score of 0.4 (by default), a PPI network containing a total of 49 nodes with 68 interactions were identified (visualized by Cytoscape 3.5.1 software in Figure 6(A)). Subsequently, by adopting the CenSiCaPe plugin in Cytoscape, 13 proteins (CETN2, PPP2R1A, HIST3H3, TUBB1, ACTB, OAS1, ACTC1, CANX, ACP1, UFD1L, H6PD, ITPA, EXOSC4) were filtered out from the network with a centrality degree greater than the threshold value of 2.7755 (Figure 6(B)). These were therefore considered the hub proteins.

| Gene symbol | Name | p value | Fold change | Regulation |
|-------------|------|---------|-------------|------------|
| SPHK1       | Sphingosine kinase 1 | 0.0484 | 1.582 | Up |
| SLC9C1      | Solute carrier family 9, isoform 10 | 0.0428 | 1.579 | Up |
| OR1M1       | Olfactory receptor 1M1 | 0.0358 | 1.494 | Up |
| APDC1       | Apolipoprotein C-1 | 0.0324 | 1.452 | Up |
| TUBB1       | Tubulin beta-1 chain | 0.0316 | 1.420 | Up |
| OAS1        | 2’-5’ oligoadenylate synthetase | 0.0090 | 1.380 | Up |
| SEC24A      | Protein transport protein Sec24A | 0.0060 | 1.376 | Up |
| ITFA        | Inosine triphosphate pyrophosphatase | 0.190 | 1.367 | Up |
| RNF31       | E3 ubiquitin-protein ligase RNF31 | 0.019 | 1.346 | Up |
| GALNT2      | Polypeptide N-acetylgalactosaminyltransferase | 0.041 | 1.329 | Up |
| STK39       | Serine threonine kinase 39 isoform D | 0.0316 | 1.572 | Down |
| PPP2R1B     | Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta | 0.0205 | 0.568 | Down |
| EXOSC6      | Exosome complex component MTR3 | 0.0117 | 0.512 | Down |
| TBC1D9B     | TBC1 domain family member 9B | 0.0280 | 0.505 | Down |
| KCTD15      | BTB/POZ domain-containing protein | 0.0023 | 0.497 | Down |
| PALD1       | Paladin | 0.0116 | 0.460 | Down |
| KRT75       | Keratin, type II cytoskeletal 75 | 0.0168 | 0.443 | Down |
| NAALADL2    | Inactive N-acetylated-alpha-linked acidic dipeptidase-like protein 2 | 0.0176 | 0.433 | Down |
| KRT5        | Keratin, type II cytoskeletal 5 | 0.0085 | 0.407 | Down |
| KRT76       | Keratin, type II cytoskeletal 2 oral | 0.0245 | 0.373 | Down |
proteins. Within the entire PPI network, two interaction modules were mined with use of the MCODE plugin in Cytoscape by setting the cutoff degree to 2 (Figure 6(C,D)). A BP enrichment analysis performed on the two modules showed that Module 1 was more enriched in processes related to DNA assembly and anti-viral activities, whereas Module 2 was more enriched in processes involving metabolism and catabolism (Figure 6(E)).

**Discussion**

During clinical application, local hyperthermia has been shown to be particularly more advantageous in treating massive or multiple HPV infected lesions [8,18,19] by interfering with only one specific lesion region or component [19], we have speculated that the efficacy of local hyperthermia may be due to its immunologic effects against HPV. Findings from a series of experiments conducted in our laboratory [11,20–23] have revealed that local hyperthermia not only edited the HPV genome and induced apoptosis of HPV-infected keratinocytes, but also activated multiple anti-HPV cytokines and promoted maturation of Langerhans cells, which may induce strong systemic anti-HPV immunity to result in the alleviation of massive or multiple lesions present throughout the body. The proteomic and bioinformatic assays carried out in this study further confirmed these speculations that hyperthermia may work through inhibition of biochemical processes related to energy and nucleic acid metabolism as well as keratinocyte differentiation, and stimulation of processes involved with antigen presentation and anti-virus activity.

Metabolism modulation is one of the most important strategies utilized by HPVs to infect and transform host cells. HPV oncoproteins, such as E6 and E7, affect
Figure 4. Gene ontology and KEGG enrichment analysis of DEPs. (A) The top ten gene counts of GO-BP (top 10 bars), MF (middle 10 bars) and CC (bottom 10 bars) terms are illustrated on the left side, with respective enrichment scores ($-\log_{10}$ (p values) × 10) shown on the right. (B) The top ten gene counts of KEGG terms are illustrated on the left side, and their respective enrichment scores ($-\log_{10}$ (p values) × 10) are shown on the right.

Figure 5. Clustering analysis of the DEPs and GO-BP enrichment of clusters. (A) The optimal cluster number was evaluated with use of gap statistics. Dashed line indicates the optimal k value of 2. (B) K-means clustering analysis of DEPs applying optimal k value revealed 2 distinct clusters, with triangles indicating Cluster 1 and spots Cluster 2. (C) Hierarchical clustering analysis of DEPs applying the optimal k value. 'C' refers to the 37 C control groups whereas 'T' refers to the 44 C hyperthermia groups. Gene symbols of DEPs are illustrated on the right side. (D) Biological process terms of the top 15 gene count in the GO-BP enrichment analysis for Cluster 1 (top 15 bars on the left) and Cluster 2 (bottom 15 bars on the left). Bars on the right represent respective enrichment scores ($-\log_{10}$ (p values) × 10).
metabolic enzymes or related signaling pathways to enhance energy production in keratinocytes [24], elevate protein synthesis [25] and promote cellular proliferation [26]. Besides, the complete differentiation and keratinization procedure of keratinocytes also plays an additional pivotal role in the life cycle of HPVs [27]. By coupling with normal epithelium developmental processes, HPVs can impede regular immune responses [28], and therefore escape identification and elimination by intrinsic and innate immunities. On the proteomic level, we found that hyperthermia reduced expression of energy metabolic enzymes such as Galactose-1-phosphate uridyl transferase (GALT) and GDH/6PGL endoplasmic bifunctional protein (H6PD), as well as several keratins (Keratin 5, Keratin 27, Keratin 75 and Keratin 76) responsible for cornification. By doing so, hyperthermia may likely obstruct the normal proliferation and differentiation processes of HPV-infected keratinocytes and, in this way, interfere with HPV life cycle.

On the other hand, the study revealed that multiple biological processes involved with viral responses were enhanced and proteins with anti-viral activities such as 2'-5'-oligoadenylate synthase 1 (OAS1), Interferon-induced GTP-binding protein Mx1 (MX1), Barrier-to-autointegration factor 1 (BANF1), Calnexin (CANX) as well as the AP-1 complex subunit sigma-1A (AP1S1) were induced by hyperthermia. These proteins have been reported to be crucial for anti-viral responses involving viral genome degradation [29,30], interferon activation [31,32], viral replication inhibition [33,34] as well as antigen presentation [35,36]. Therefore, we proposed that by induction of these key factors, hyperthermia has the potential to interfere with HPV DNA replication and expression as well as HPV antigen presentation and, in this way, boost the immunologic clearance of HPV infections.

Figure 6. Protein-protein interactions of DEPs. (A) PPI network of DEPs visualized by Cytoscape. Circles indicate up- or down-regulated DEPs. Edges connecting the circles indicate protein-protein interactions, with thickness of the edges representing confidence of the bonds. (B) Hub proteins identified by CentiScaPe were highlighted in the PPI network. (C and D) Interaction module 1 (C) and module 2 (D) mined from the PPI network with use of MCODE analysis. (E) Biological process terms of the top 15 gene count in the GO-BP enrichment analysis for Module 1 (top 15 bars on the left) and Module 2 (bottom 15 bars on the left). Bars on the right represent respective enrichment scores (−log_{10}(p values) × 10).
Usually, proteins within the same interaction module possess similar functions [37], and the GO-BP enrichment analysis of the two major interaction modules mined from the PPI-network revealed a highly similar functional involvement as compared to those identified by clustering, indicating that the interaction modules mined from the PPI network were crucial to the overall proteomic changes that result from hyperthermia, and therefore warranted further attention and more detailed investigations.

By combining proteomic and bioinformatics analyses, results a considerable amount of new and interesting information regarding local hyperthermia therapy against CA has ensued. We will be more concentrated on the processes involving energy generation and cornification control of keratinocytes as well as proteins with anti-viral activities identified by this work in our future studies. However, it should also be noted that both expression patterns and responsiveness of some DEPs to hyperthermia showed considerable variation among different CA samples. Hence, in order to obtain more robust results, an increasing number of samples should be included in any further investigations.

Overall, we believe these current results, in particular, those derived through analyzing the hub proteins and their potential interactions, provide a foundation for future avenues of research into the detailed mechanisms involved with the efficacy of local hyperthermia and will guide us to improve the implementation of this promising clinical therapeutic method against cutaneous HPV infection.

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Disclosure statement

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