Cellular and Molecular Mechanisms of Photodynamic Hypericin Therapy for Nasopharyngeal Carcinoma cells

Xiaoli Wang, Yi Guo, Shu Yang, Caihong Wang, Xuping Fu, Jinling Wang, Yumin Mao, Junsong Zhang, Yao Li

State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Science, Fudan University, Shanghai 200433, P.R.China (Y.G., S.Y., X.F., Y.M.,Y.L.)

School of Applied Chemistry and Biological, Shenzhen Polytechnic, Shenzhen, Guangdong 518055, P.R.China.(X.W., C.W., J.W.,J.Z.)
Cellular and Molecular Mechanisms of HY-PDT for NPC cells

*Corresponding authors:

Prof. Yao Li

State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Science, Fudan University, Shanghai 200433, P.R.China

Tel: +86 21 6564 2047; Fax: +86 21 6564 2502;

E-mail: yaoli@fudan.edu.cn

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**Abbreviation:** HY, hypericin; PDT, photodynamic therapy; HY-PDT, Hypericin-mediated Photodynamic therapy; LED, light emitting diode; PS, phosphatidyserine; NPC, Nasopharyngeal Carcinoma; ROS, reactive oxygen species; GSH, Glutathione; FBS, fetal bovine serum; IR, inhibition rate; DMSO,
dimethylsulfoxide; HP, Hematoporphyrin; FITC, fluorescein isothiocyanate; PI, Propidium Iodide; MTT, Methylthiazolyl diphenyl-tetrazolium bromide; DE, Differentially expressed; ETC, electron transport chain; Qi, quinone reducing center; FADD, FAS-Associated death domain-containing protein; tBid, truncated Bid.

A recommended section: Cellular and Molecular
Abstract

Hypericin-mediated Photodynamic therapy (HY-PDT) has becoming of a potential treatment for tumors and nonmalignant disorders. Some studies reported that HY-PDT could lead to apoptosis in some carcinoma cells. However, the molecular mechanism of HY-PDT remains unknown. In this study, we evaluated the molecular mechanisms of Hypericin associated with light emitting diode irradiation on poorly differentiated human nasopharyngeal carcinoma cell line CNE-2 in vitro. To comprehensively understand the effects of HY-PDT on CNE-2 cells, we detected cell viability, cell cycle, apoptosis, intracellular glutathione content and intracellular caspase (caspase-9, caspase-3 and caspase-8) activity. Furthermore, we performed genome-wide expression analysis via microarrays at different time-points in response to HY-PDT, and found differentially expressed genes were highly enriched in the pathways related to reactive oxygen species (ROS) generation, mitochondrial activity, DNA replication and repair, cell cycle/proliferation and apoptosis. These results were consistent with our cytology test results, and demonstrated that caspase-dependent apoptosis occurred after HY-PDT. Taken together, both cellular and molecular data revealed that HY-PDT could inhibit the growth of CNE-2 cells and induce their apoptosis.
Photodynamic therapy (PDT) is one of the newest advancements in the management of different microbial, viral, fungal, inflammatory disorders and a variety of cancers. Light-induced growth inhibition is used in this method. It involves the targeting of cells or tissues which have been sensitized to light by administration of a photosensitizing agent. One such agent is Hypericin (HY; 1,3,4,6,8,13-hexahydroxy-10,11-dimethyl-phenanthro[1,10,9,8-opqra]perylene-7,14-dione) (Falk, 1999), is a secondary metabolite which can be isolated from *Hypericum perforatum*, commonly known as St. Johns Wort plant. Due to its photoactive properties and low cytotoxicity, attention has been focused on its application in PDT (Okpanyi et al., 1990; Kersten et al., 1999; Agostinis et al., 2002; Roscetti et al., 2004; Kiesslich et al., 2006).

Apoptosis and necrosis are two kinds of PDT induced cell death (Fiers et al., 1999). Which pathway is induced depends on the different property of photosensitizer, type of cells, density of population, and the experimental method. Further, the method itself varies by photosensitizing agent concentration, light dose, and incubation time (Blank et al., 2002; Alvarez et al., 2003). Which pathway the cell takes to PDT induced death is organelle dependent as well. That is, plasma membrane and lysosome can lead to necrosis while mitochondrial activity can lead to programmed cell death, including both caspase-dependent and independent apoptosis (Chen et al., 2000). Caspase-dependent apoptosis includes two pathways, the extrinsic death pathway (death receptor-dependent) and the intrinsic death pathway.
(mitochondria-dependent). In the past decades, mitochondria has played an important role in initiating and executing apoptosis in several types of cells (Green and Kroemer, 2004; Bras et al., 2005; Zoratti et al., 2005).

Previous studies showed PDT associated with Hypericin could lead to apoptosis in human nasopharyngeal carcinoma cell line CNE-2 (Ali et al., 2001; Ali and Olivo, 2002), but others found it yielded necrosis (Du et al., 2003). Ali and colleagues (2002) reported photosensitization of HY could enhance CD95/CD95L expression and CD95-signaling dependent cell death in all tumor cell lines (Ali et al., 2002). This was accomplished in poorly differentiated CNE-2 cells by measuring the change of membrane potential in mitochondria, the release of cytochrome C, the activity of caspases-3, 8, as well as the status of caspase-3 specific substrate PARP (Poly [ADP-ribose] polymerase), and expression of CD95/CD95L. Thong and colleagues (2005) used PDT associated with Hypericin treated cells and reported the intracellular concentration of Ca$^{2+}$ in experimental group to be significantly higher than control (Thong et al., 2005).

Furthermore, many other reports indicated HY-PDT could lead to apoptosis in human laryngeal squamous cell carcinoma strain, Hep-2 (Sun et al., 2005), hepatoblastoma (HB), pediatric hepatocellular carcinoma cells (HCC) (Seitz et al., 2008), GH4C1 rat pituitary tumors (Cole et al., 2008), childhood rhabdomyosarcoma (Seitz et al., 2007), and esophageal cancer cells (Hopfner et al., 2003). Nevertheless these works lacked sufficient evidence regarding to the molecular mechanism for HY-PDT. Additionally, some experiments demonstrated apoptosis induced by
HY-PDT could be promoted by inhibiting p38 MAPKs and that such cell demise could be stimulated by HY-PDT associated with some other drugs (Kocanova et al., 2007; Buytaert et al., 2008; Schneider-Yin et al., 2009).

Microarray analysis has been widely employed in the detection of differentially expressed genes as well as the pathway analysis for potential molecular mechanisms (Watts et al., 2001; Sarkozi et al., 2008). Recently, Sanovic and colleagues (2009) detected an alteration of the gene expression in the human squamous cell carcinoma cell line A-431 after HY-PDT by cDNA-microarray technique (Sanovic et al., 2009). One hundred and sixty eight genes were found to be differentially up-regulated and forty-five were down-regulated. Due to the significant expression changes the following could be concluded: 1) lipoprotein receptor-mediated endocytosis could play an important role in the uptake of lipophilic Hypericin, and 2) cytoskeleton rearrangement and formation of apoptotic bodies occurred.

Although some studies about apoptosis induced by HY-PDT, there were neither genome-wide expression profile studies nor systematic molecular evidence could reveal the mechanisms of apoptosis in CNE-2 cells subjected to HY-PDT. To gain insight into the complex molecular mechanisms of this therapy, we conducted both cytology tests and genome wide expression analysis of Hypericin associated with light emitting diode (LED) irradiation of human CNE-2 cells line in vitro. We found numerous features with respect to molecular pathways which involved in various aspects of cell proliferation, DNA repair and apoptosis.
Methods

Cell Culture: Human nasopharyngeal Carcinoma cell lines CNE-2 were purchased from Shanghai Cell Biology Institute of Chinese Academy of Science. Cells were grown at 37°C, 100% humidity, 95% air, 5% CO₂ and fed with 10% fetal bovine serum (FBS), and RPMI 1640 (Gibco, America). RPMI 1640 was supplemented with 1% L-glutamine, 100μg/ml penicillin and 100μg/ml streptomycin.

Drug Treatment: In cell viability experiment, CNE-2 cells were seeded in 96-well plate (10⁴ cells/100μl media/well) and were cultured as before. After 24 h, Hypericin (Alexis, America), which was dissolved in DMSO, was added to increasing concentrations (0.04, 0.08, 0.12, 0.16 and 0.20μg/ml). Cells were incubated for 6 h in the dark, and then exposed to yellow LED for Hypericin and red LED for Hematoporphyrin (HP) irradiation for 90 min PDT. The light energy was 5.67 J/cm². After drug treatment, cells were incubated in the dark.

Afterwards, we established the following experimental group and control groups: treatment of 0.20μg/ml HY and light irradiation as the experimental group (group A); treatment of 0.20μg/ml HY without irradiation as the negative control group (group B). In addition, we added one or two extra negative controls in different experiments: treatment of irradiation without HY (group C) and treatment without HY or irradiation (group D).

Cell Viability: After drug treatment, cells were incubated in the dark for an additional 20 h. Meanwhile, the group which contained DMSO only and the groups which contained HP in increasing concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5,
4.0μg/ml) were designated as the blank control and positive control respectively. Cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. After 20 h incubation, medium was removed and cells were washed with PBS (pH7.4). Then an additional 100μl of medium containing MTT (0.5mg/ml) without FBS was added and cells were incubated 4 h in dark. Afterwards, the medium was decanted carefully, dissolved in formazan in 100μl of DMSO and absolute ethanol (1:1) with agitation for 15 min. Absorption was measured on a Spectramax M2 microplate reader (Molecular Devices, USA) at 570 nm. Inhibition rate (IR) (%) = (1-average A570 of the experimental group/average A570 of the control group) ×100%. IC50 was calculated using the Bliss method.

**Cell Cycle:** Cell cycle test kits (CycleTest Plus DNA Reagent Kit, Becton Dickinson, USA) were used according to the manufacturer's instructions at 20 h after light irradiation. Cells were collected by centrifugation at 400×g for 5 min and then removed supernatant. We then re-suspended pellet in 250μl solution A, containing trypsin, and incubated for 15 min. Next we added 200μl solution B, containing trypsin inhibitor and RNase A, and then carefully mixed. After 15 min incubation, 200μl solution C, containing PI, was added and incubated at 4°C for 15 min in dark. Finally, each sample was filtered by 50μm aperture nylon membrane. The results were detected by FACSCalibur Flow Cytometry (Becton Dickinson, USA). After using CellQuest software to collect about 20,000 cells, cell cycle was analyzed by ModFit LT 2.0 software.

**Apoptosis:** Cells in group A and B were incubated in the dark for additional 18 h,
28 h and 48 h. Then apoptosis was determined by apoptosis test kit (APO-BRDU Kit, Becton Dickinson, America) according to the manufacturer's instructions. Cells were collected and re-suspended in 0.5ml of 1×PBS. Next we added 5 ml 1% paraformaldehyde. Cells were then incubated on ice for 15 min. We then re-suspended cells in 0.5ml 1×PBS and centrifuged at 400×g for 5 min. After this, we re-suspended cells in 0.5ml 1×PBS. Next 70% ethanol was added and cells were incubated at -20°C overnight. We then centrifuged cells and re-suspended them in 1ml Wash Buffer. After another round of centrifugation, cells were re-suspended in 50μl DNA marker solution, and incubated at 37°C for 60 min. We next added 1ml RNase Buffer, and each tube was incubated for 10 min, and centrifuged at 300×g for 5 min. We next removed supernatant, and re-suspended cells in 0.1ml fluorescent-labeled anti-Brdu antibody. After 30 min incubation at 37°C, we added 0.5 ml PI (0.25%)/RNase A solution, and incubated again at 37°C for 30 min in dark. The results were detected by Flow Cytometry.

**Intracellular Glutathione Content:** Cells in group A, B, C and D were collected by centrifugation. Content of intracellular glutathione (GSH) was determined by test kit (ApoAlert Glutathione Detection Kit, Clontech, Japan) according to the manufacturer's instructions. Cells were collected and re-suspended in 10ml of fresh RPMI medium containing 10% FBS. Cells were collected by centrifugation at 700×g. The pellet was re-suspended in 1 ml ice-cold 1×Cell wash buffer. We then transferred re-suspended pellet into a 1.5ml micro-centrifuge tube, and centrifuged at 700×g for 5 min. We then re-suspended pellet in 100μl ice-cold 1×Cell Lysis Buffer and incubated
for 10 min on ice, then centrifuged at maximum speed using a tabletop centrifuge for 10 min. Following, we added 2μl of 100mM Monochlorobimane (MCB) to each supernatant. Herein, we prepared a negative control sample by adding 2μl of MCB to 100μl of 1×Cell Lysis Buffer. All samples were incubated at 37°C for 15 min. The fluorescence was detected by Fluorometer (Varian Cary Eclipse Fluorescence Spectrophotometer) at 395 and 480 nm.

**Intracellular Caspases, Caspase-9, Caspase-3 and Caspase-8 Activities:** Cells in group A, B, C and D were collected by centrifugation. According to the manufacturer's instructions, the overall caspases, caspase-9, caspase-3 and caspase-8 activity were determined by the appropriate caspase test kit (CaspGLOW Fluorescein Caspase Staining Kit, BioVision, USA), respectively. Aliquots of 300μl from cell culture from each group were transferred to eppendorf tubes. We then added 1μl of FITC-VAD-FMK, FITC-LEHD-FMK, FITC-DEVD-FMK and FITC-IETD-FMK, respectively, into each tube and incubated for 0.5 h at 37°C with 5% CO₂. Cells were then centrifuged at 3000 rpm for 5 min and supernatant removed. We then re-suspended cells in 0.5 ml of Wash Buffer, and centrifuged again. This step was repeated once. The results were detected by Flow Cytometry.

**Transcriptional Analysis of time course in response to HY-PDT:** After both treatment (0.20μg/ml HY) and irradiation, cells were incubated in the dark for 0 h, 2 h, 6 h, 12 h and 20 h as experimental groups. Cells in HY treatment without irradiation were incubated in the dark for 20 h as the control group. Total RNAs were isolated from the cultured cells in both experimental and control groups, and hybridized to
Illumina Sentrix Human WG-6_V2 expression BeadChip arrays separately according to the manufacturer's instructions.

Microarray data analysis: Gene expression measures were available at GEO (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20134). The data were extracted using BeadArray scanner and analyzed by BeadStation software provided by the manufacturer. Genes, whose DiffScore was above 13 when comparing to the control, were defined as differentially expressed genes. The Cluster program (Eisen et al., 1998) was used to cluster the candidate genes into a hierarchical tree. Pathway analysis was performed with GenMAPP 2.0 software (Dahlquist et al., 2002; Doniger et al., 2003). Significantly altered pathways were defined when their Z score were above 1.96.

Statistics: Means and standard deviations of individual groups (n=3) were calculated. P-values were assessed by performing two-sided student’s t test.
Results

Cell Viability: Irradiated with LED light, the cell viability showed significant differences between the cells added with increasing concentrations of HY (0.04, 0.08, 0.12, 0.16 and 0.20 μg/ml) and blank control ($P<0.01$, two-sided t-test). When the final concentration of HY reached 0.16 μg/ml, IR was more than 90% (Fig. 1) with IC$_{50}$ 0.049 μg/ml. In the presence of HY without irradiation, cell viability was slightly inhibited (Fig. 1). However, there was a difference between treatment with irradiation and non-irradiation at the same concentration of HY ($P<0.01$) (Fig. 1). As illustrated, the HY-PDT was dose dependent and the IR of the irradiation group was much more than the non-irradiation group. Furthermore, application of Hematoporphyrin and irradiation with red LED light yielded inhibition of CNE-2 ($P<0.01$) (Fig. 2) with IC$_{50}$ 0.650 μg/ml. The IC$_{50}$ of HY was 13 times less than that of HP’s.

Cell Cycle: Our data detected by Flow Cytometry showed cell proportion at the G1 phase in the experimental groups was lower than that in the control groups. Meanwhile, the cell proportions of S and G2 phase were higher in experimental group than those in controls (Table 1).

Apoptosis: Our data detected by Flow Cytometry showed there was a small amount of apoptotic cells in the group of HY without irradiation, and the proportion of apoptosis cells reached 22.37% after 48h culture. However, the apoptosis ratio of HY-irradiation group was markedly higher than non-irradiation group. The ratios were 72.19% and 92.24% respectively after 28 and 48 h (Table 2). This outcome was consistent with our externalization of membrane phosphatidylserine (PS) test (data not
Intracellular Glutathione (GSH) Content: The glutathione content was markedly decreased in group A. In contrast, the content in group B and C had slightly decreased ROS compared with group D. (Fig. 3). Since GSH was depleted to reduce ROS, this result suggests ROS were largely generated during HY-PDT.

Intracellular Caspase Activity: The activity of total caspases, caspase-9, caspase-3, and caspase-8 in the CNE-2 cells were detected by Flow Cytometry, and were activated in group A only. In contrast, there was no indication of total caspase, caspase-9, caspase-3, or caspase-8 activity in the other three controls (group B, C and D) (Table 3).

Differentially expressed (DE) mRNAs: The cellular responses of HY-PDT treatment suggested many genes contributed to these phenotypes. We followed the transcriptional profile of CNE-2 with HY-PDT through an extended time course, with samples taken from cultures 0, 2, 6, 12, 20 h after 1.5 h exposed to LED irradiation.

Genes which differentially expressed in at least one time point in response to the HY-PDT treatment are defined as DE mRNA. We obtained 5619 DE mRNAs, in which 2889 mRNAs were up-regulated, and 2730 mRNAs were down-regulated. Meanwhile, 370 mRNAs remained differentially expressed throughout the entire time course, in which 140 genes were induced and 230 genes were repressed. Detailed gene information is listed in supplements (Table S1, Table S2).

We have applied hierarchical clustering to the DE genes and samples separately (Fig. 4). The temporal expression at time point 0 (without incubation immediately
after irradiation) is quite different from those in the following incubation. Moreover, among all the time points, we observed the largest group of DE genes at 0 h time point, 1775 genes up-regulation and 1451 genes down-regulation, and the second largest group is at 20 h time point.

**ROS activation, damage of mitochondrial membrane and DNA:** Next, we used GeneMapp to analyze the pathway involved in HY-PDT treatment based on the DE gene at each time-point respectively (Table 4). Detailed pathway information is listed in the supplement (Fig. S1-S6, Table S3-S7). Noticeably, there were more altered pathways at 0 h time-point than those at the following time points.

The oxidative stress pathway was significantly induced immediately after irradiation (0 h time point), consistent with glutathione test. We suggest ROS was produced in the presence of HY-PDT (Fig. S3).

In addition, mitochondrial inner membrane proteins were induced to higher mRNA levels during the early time course (Fig. S4). This is consistent with the theory that one target of HY-PDT is the mitochondrial electron transport chain (ETC), and more precisely that the focus of damage is at the quinone reducing center (Qi) of complex III (Theodossiou et al., 2008; Theodossiou et al., 2009).

Pathways involved in helicase activity, DNA replication and DNA repair were also induced at time-point 0. Helicase activity remained for a longer time to cause DNA unwinding and chromatin disassembling in the end of time course.

**Cell cycle and cell proliferation:** DNA microarray also revealed some interesting expression patterns of the cell cycle pathway. They were altered in the early time
points in response to HY-PDT, consistent with the cell cycle test (Table 1). This suggests the cells were enriched in S and G2 phases for repairing the damaged DNA (Fig. S2). Meanwhile, cellular metabolism was negatively regulated.

The pathway of negative regulation of cell proliferation was significantly activated during the entire time course, confirming the result of MTT assay that HY-PDT can inhibit cell proliferation (Fig. S1).

**Apoptosis:** Apoptosis pathways were significantly altered in the entire time course. Change of mitochondrial membrane indicated that the intrinsic cell death pathway was activated. Further, the Fas extrinsic cell death pathway was significantly activated during the early time course, in which FADD in death-inducing signal complex (DISC) was up-regulated. Moreover, extrinsic cell death pathway modulation by HSP70 appeared markedly different at the 6 h time-point, which is suggestive of extrinsic cell death pathway activation. These results were confirmed by detection of capase-8 activity (Table 3).

It should be noticed that most of caspases were not significantly induced in mRNA level during the treatment, whereas caspase test showed the activation of caspases. Many cysteine-type endopeptidases involved in proteolysis were induced immediately after irradiation, such as members of ubiquitin-specific processing protease family, USP8, USP10, USP13, USP30, USP38, USP39, USP49 etc, indicating that the cellular proteolysis was significantly promoted by HY-PDT.

**Other pathways related with different cellular behaviors:** HY-PDT can also induce an inflammatory response in the middle of time course. Pathways related to
intracellular structures such as the lysosome, Golgi apparatus, and cytoskeleton also showed alteration compared to the control, which indicated that HY-PDT might induce the instability of intracellular structure. Finally, G-protein-mediated signaling pathways were altered at 20 h time point.
Discussion

Theoretically, HY-PDT treatment could result in both ROS overproduction and disruption of the homeostasis of the inner mitochondrial membrane, and then damage the outer membrane of mitochondria. This damage would decrease the membrane potential and change the permeability of the outer mitochondrial membrane which is related to both apoptosis and necrosis. Reportedly, HY-PDT could induce apoptosis in several kinds of carcinoma cells (Ali et al., 2001; Ali and Olivo, 2002; Hopfner et al., 2003; Sun et al., 2005; Seitz et al., 2007; Cole et al., 2008; Seitz et al., 2008). Apoptosis was detected in HY-PDT treatment cells, and was promoted using this therapy.

Although some reports showed there was an obvious effect on CNE-2 using PDT (Ali et al., 2001; Ali et al., 2002; Ali and Olivo, 2002), our paper tried to explain the molecular mechanism of HY-PDT in inhibition of viability and induction of apoptosis in vitro, using LED light source to replace the expensive laser.

Here we found a dose-dependent inhibition of CNE-2 cell proliferation by HY-PDT in vitro. Importantly, HP has been showed to more side effects such as retention in the body as long as 4 weeks. Thus, patients need to avoid light during this period of time, and higher concentrations of HP increase the photoallergic reaction. According to our data, HY as an ideal photosensitizer had more advantages than HP. HY’s IC_{50} (0.049 μg/ml) was 13 times less than HP’s (0.650 μg/ml).

Further, the apoptotic ratio significantly increased with prolonging time in the irradiation group. At 28 h there was 72.19% and at 48 h was 92.24% compared with
the non-irradiation group. This result showed the significant inhibition of cell proliferation with irradiation in CNE-2 cells. Further it is in agreement with several reports (Seitz et al., 2008; Ferenc et al., 2009; Mikes et al., 2009), showing HY had the ability to inhibit biological activity with irradiation.

According to the previous studies, ROS were largely produced during PDT (Buytaert et al., 2007). With this in mind, we further tested the intracellular glutathione content. A large number of GSH was depleted during this therapy, so we deduced ROS was induced and GSH was used to eliminate ROS. In this case, we hypothesized the photogenerated ROS would induce release of cytochrome C and activation of the caspase cascade.

In order to understand if or which phase cells were or were arrested, we characterized the cell cycle and found the cells of S and G2 phase increased. According to the previous studies, S phase was a sensitive period for chemotherapeutics, and drugs could inhibit DNA synthesis and the growth of carcinoma cells by arresting this phase. Thus, we suggest HY-PDT likely affects DNA synthesis and replication.

To determine which kind of apoptosis pathway was stimulated, we tested the activation of overall caspases. Compared to the control group, caspases were markedly activated in the HY-PDT group. We found that cell death caused by HY-PDT therapy was due to caspase-dependent apoptosis.

Caspase-dependent apoptosis signaling pathways have been found in two sorts, mitochondria dependent (intrinsic signal) and extrinsic signal (such as TNF and Fas...
ligand) dependent. Some reports showed mitochondria were not only the target of HY, but also was first target of photodynamic therapy (Theodossiou et al., 2008), acting on mitochondria, releasing cytochrome C and changing DNA fragments and cell morphology.

We further detected the activity of caspase-3, 8, and 9 respectively. Results showed all of them were activated. The activated caspase-3 and 9 indicated that intrinsic cell death pathway was activated by HY-PDT. Unexpectedly caspase-8 was also activated during HY-PDT, which suggested that extrinsic cell death pathway also contributed to this therapy.

Analysis of the genes of HY-PDT induction should offer insights into the response of CNE-2 cells and resulting apoptosis. Therefore we detected the gene expression profiling at the transcriptional level at 5 different time-points in response to HY-PDT.

By analysis of DE genes related to HY-PDT and pathways involved in biological processes, we found that DE genes were highly enriched in the pathways involved in ROS, mitochondria, DNA replication and repair, cell cycle, cell proliferation, and apoptosis. Microarray analysis results were consistent with our cytology test results, and evidently demonstrated mitochondria-dependent apoptosis occurs by HY-PDT. In addition, we detected the differential expression of genes in the FAS pathway and stress induction of HSP regulation, which confirmed the existence of extrinsic cell death (Fig. S6). The reason for phenomenon remains unknown. According to our detected FADD up-regulation and caspase-8 activation, we deduced the Fas pathway was not activated directly by Fas ligand, but by amplification of internal signaling
through the recruitment of more death domain containing protein (FADD) and initiator caspase-8. Caspase-8 subsequently led to the proteolytic activation of the main effector caspase-3/7 and generated truncated Bid (tBid), which could, in turn, bind to Bcl-2, inhibiting its anti-apoptotic function.

Interestingly, most caspases were not significantly induced in mRNA level during the treatment, whereas caspase test showed the caspases activity were activated. This phenomenon suggested that caspase-3, 8, and 9 were activated via promoting of the cleavage of inactive pro-caspases in response to HY-PDT induction.

Among all the time points, we observed that DE gene at 0 h time point was the largest, 1775 genes of up-regulation and 1451 genes of down-regulation. The reason was the cells at 0 h time point had been irradiated with HY-PDT for 90min. During this period of time, cells dramatically responded to photodynamic pressure and had enough time to change their expression behaviors to adapt to the environment rapidly. Please refer to supplementary table (Table S3) for detail information of 0 time point pathway analysis. Interestingly, differentially expressed pathways in this time point included the majority of organelle pathways which altered in other time points, such as helicase activity, DNA replication, response to DNA damage, cell proliferation, cell cycle, apoptosis to stimulus, oxidative stress, mitochondrial membrane, caspase, Fas pathway, stress induction of HSP regulation and Golgi apparatus. With the depriving of PDT and the prolonging of culture time, cells were no longer yielded to the photodynamic pressure, and became moderate gradually.

In conclusion, from the results of these experiments, HY-PDT induces the
generation of ROS, which attacks the mitochondrial membrane and DNA in both
direct and indirect way. In addition HY-PDT activates Cysteine-type endopeptidase,
which inhibits the cells growth, and induces apoptosis. We propose the apoptosis
induced by HY-PDT is subserved by both the mitochondria-dependent intrinsic
pathway, as well as the activation of the extrinsic pathway.
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profiling of human squamous cell carcinoma cells during the apoptosis process induced by photodynamic treatment with hypericin. *Int J Oncol* **35**:921-939.


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§These two authors contribute equally to this paper.
Legends of Figures

Fig. 1 Inhibition rates of cell viability test of HY in treatment with or without irradiation by MTT Assay. Cells were treated with increasing concentrations (0.04, 0.08, 0.12, 0.16 and 0.20μg/ml) of HY with or without irradiation, and incubated an additional 20 h in dark. Then cell viability was tested by MTT assay. The mean and the standard deviation of the inhibition rate for each HY concentration are shown as a dot and vertical line, respectively. (-●- HY with irradiation, -□- HY without irradiation)

Fig. 2 Inhibition rates of cell viability test of HP treatment with irradiation by MTT assay. Cells were treatment with increasing concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0μg/ml) of HP and irradiation, and incubated an additional 20 h in dark. Then cell viability was tested by MTT assay. In our data, cells viability was inhibited by HP-PDT. When the final concentration of HP reached 1.5μg/ml, IR was more than 90%. The mean and the standard deviation of the inhibition rate for each HP concentration are shown as a dot and vertical line, respectively. (-■- HP with irradiation)

Fig. 3 Results of intracellular glutathione content test. Cells of group A, B, C and D were collected by centrifugation. Content of intracellular glutathione was determined according to the manufacturer's instructions by test kit. Here we prepared a sample without cells by adding 2μl of MCB to 100μl of 1× Cell Lysis Buffer as negative control. The fluorescence was detected by Fluorometer at 395/480 nm. The mean and the standard deviation of the fluorescence test result for each HY concentration are
shown as a bar and vertical line, respectively.

Fig. 4 Cluster analysis of 5 different time-point gene expression compared with control respectively. The full heatmap for the unsupervised clustering of CNE-2 lines, with the indicated time-points. Each horizontal row indicates a single time point different gene expression from the indicated treatment group. Genes represented with color have a DiffScore of 13 or more. Red indicates genes up-regulated relative to control, whereas green indicates genes that were down-regulated. Black indicates that the gene expression was not changed relative to control.
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<th>G1 (%)</th>
<th>S (%)</th>
<th>G2 (%)</th>
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<td>Neither HY nor irradiation</td>
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TABLE 2

Effect of HY on CNE-2 Apoptosis

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<td>HY without irradiation</td>
<td>1.74</td>
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TABLE 3
Results of Caspase Activity Test

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<td></td>
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<td>M1 (%), M2 (%)</td>
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<td>(Group A)</td>
<td>(Group B)</td>
<td>(Group C)</td>
<td>(Group D)</td>
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<td>97.79, 2.04</td>
<td>96.92, 2.99</td>
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<td>Irradiation without</td>
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<td>96.42, 3.58</td>
<td>96.1, 3.71</td>
<td>97.87, 1.97</td>
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<td>Neither HY nor</td>
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<td>99.17, 0.71</td>
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<td>98.93, 1.02</td>
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<td>(Group D)</td>
<td>(Group D)</td>
<td>(Group D)</td>
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M1: cells not containing FITC; M2: cells containing FITC
TABLE 4

Significant altered pathways during the time course

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<th>Function</th>
<th>MAPP pathway Name</th>
<th>0 h</th>
<th>2 h</th>
<th>6 h</th>
<th>12 h</th>
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<td>P-value</td>
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</table>

* "-" Stands for Z score<1.96, with no significant change
Fig. 1

Inhibition rate (%) vs. HY concentration (μg/ml)

- ● HY with irradiation
- ● HY without irradiation

Error bars indicate standard deviation.
Fig. 3

- Neither HY nor irradiation (group D)
- HY without irradiation (group B)
- Irradiation without HY (group C)
- Both HY and irradiation (group A)
- Negative control (no cells)

480 nm fluorescence test result vs. HY concentration (0.20 μg/ml)