Running title: HPV genotypes and ATAD3A in UCC

Human papillomavirus infection and expression of the ATPase family, AAA domain containing 3A, a novel anti-autophagy factor, in uterine cervical cancer

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Abstract

Our aim was to determine the association of human papillomavirus (HPV) infection with expression of the ATPase family, AAA domain containing 3A (ATAD3A), an anti-autophagy factor, in uterine cervical cancer (UCC). HPV genotype was determined by an Easychip HPV blot assay. ATAD3A expression was determined by an immunohistochemical staining. High-risk HPV (hrHPV) was detected in 184 (88.9%) of UCC cases. ATAD3A expression was detected in 164 (79.2%) of UCC cases. Significant correlation was found between ATAD3A expression and the presence of hrHPV ($p < 0.001$), FIGO stage ($p = 0.014$), lymph node involvement ($p = 0.001$), c-MET expression ($p < 0.001$), interleukin-8 ($p = 0.03$) and patients' survival ($p = 0.0016$). Interestingly, silencing of E6/E7 expression decreased ATAD3A expression and cell survival. Moreover, knockdown of ATAD3A (ATAD3A^{kd}) expression or addition of resveratrol, on the other hand, increased cellular autophagy and apoptosis and reduced drug resistance. Resveratrol reduced ATAD3A expression, and increased abrasion of mitochondrial outer membrane as well as numbers of autophagosomes, the phenomena that were frequently found in ATAD3A^{kd} cells. In conclusion, our results showed that HPV infection correlated with the increase of ATAD3A expression and drug resistance in UCC. The persistent HPV infection may stabilize ATAD3A expression to inhibit cell autophagy and apoptosis as well as to increase drug resistance.

Key words: high risk HPV, ATAD3A, drug resistance, autophagy, prognosis

The abbreviations used are: HPV, human papillomavirus; hrHPV, high-risk HPV; ATAD3A, the ATPase family, AAA domain containing 3A; UCC: uterine cervical cancer

Introduction

Uterine cervical cancer (UCC) is the most prevalent cancer, and the fifth leading cause of cancer death in Taiwanese women (1). High incidence and mortality of the disease was in part due to the conservative attitude toward Papanicolauou smear, by which although the disease could be detected at the early stages the method was considered intrusive, and in part due to the high infection rate of human papillomavirus (HPV) among the diseased women (2).

HPV infection is a potential etiologic cause for UCC development (3, 4). Among 95 known HPV (5), genotypes 16, 18, 52 and 58 are categorized as high-risk and most frequently detected in the Taiwanese UCC patients (6-9). Carcinogenicity of the high risk HPV is closely associated with E6 and E7 proteins, which can respectively inhibit activities of tumor suppressors, p53 and retinoblastoma protein (pRB), to interrupt cell growth regulation (10). Besides carcinogenesis, persistent HPV infection further correlates with tumor recurrence, cancer cell radio-sensitivity and the resulting poor prognosis (11-13).

Oncogene activation is also crucial for tumorigenesis and cancer progression; in particular, genes that are associated with disease advancement and, possibly, resistance to irradiation and anticancer chemotherapeutics. By using differential display, we have identified overexpression of dihydrodiol dehydrogenase (DDH) in non-small cell lung cancer (14). Using microarray, we further found that DDH was overexpressed in cisplatin-resistant ovarian cancer cells (15). Clinically, patients with elevated DDH have higher tumor recurrence rate and lymph node metastasis, and the tumor is more resistant to cisplatin-based chemotherapy (16). Interestingly, DDH overexpression has also been identified in the UCC (6). Nevertheless, since DDH does not directly inactivate anticancer chemotherapeutics, the pro-tumorigenic effects are yet to be clarified. Recently, using suppression subtractive hybridization, oligonucleotide microarray, hierarchical clustering and subsequent gene pool

analysis, we identified several interesting genes, such as dynamin-related protein 1 (DRP1) (17), mitofusin 2 (Mfn-2) and ATPase family, AAA domain containing 3A (ATAD3A) (18),which were associated with drug resistance of lung cancer cells. DRP1 and Mfn-2 are GTPases, and ATAD3A is an ATPase. Interestingly, serum deprivation increased protein levels of ATAD3A and cisplatin resistance. Silencing of ATAD3A expression, however, increased cisplatin sensitivity, mitochondrial fragmentation and cellular autophagy, suggesting that ATAD3A could be an anti-apoptotic factor, which was essential for maintaining the mitochondrial integrity (18).However, the role of ATAD3A expression in UCC has not been investigated.

In this study, we determined the genotypes of high-risk HPV by an Easychip HPV blot assay and ATAD3A expression by an immunohistochemical staining in the UCC specimens. Relationship between ATAD3A expression and clinicopathological parameters as well as prognostic significance of ATAD3A in UCC patients was statistically evaluated. Correlation ATAD3A expression and drug resistance was further assessed in the *in vitro* experiments.

Materials and methods

Patients and tissue samples

From September 1995 to June 2004, samples from 207 consecutive patients who were diagnosed having UCC were collected. Staging of the disease was performed according to the International Federation of Gynecology and Obstetrics (FIGO) classification. Medical Ethical Committee has approved the protocol, and the written informed consent was obtained from every patient before surgery. All patients had undergone surgical resection and radical lymph nodes dissection. Tumor size, lymph node number, differentiation, vascular invasion and mitotic number were also evaluated. Patient with lymph node involvement and those with distant metastasis were treated with chemotherapy (19). Following treatment, all patients were routinely followed every 3 to 6 months in the Out-Patient Department. Tumor recurrence and metastasis were identified when blood examination, biochemical studies, abdominal sonography, whole body bone scan and computerized tomography scans of abdomen showed any suspected evidence of the disease. Tissue arrays of UCC specimens from 205 American patients (US Biomax, Inc., Rockville, MD, USA) were used to compare ATAD3A expression between the Chinese and the American patients.

HPV genotyping by Easychip HPV blot assay

Easychip HPV blot assay was used to determine the genotype of HPV infection in pathological sections (7, 8). Briefly, DNA extracted from paraffin sections was amplified in a polymerase reaction using HPV-specific MY11/GP6+ consensus primers.7 The GP6+ was biotin-labeled at the 5’ end. The amplified DNA fragments were hybridized to an Easychip® HPV blot membrane, which contained 39 genotypes of HPV oligonucleotides (King Car Food Industrial Co. Ltd, Taiwan). The membrane was probed with alkaline phosphatase-conjugated streptavidin, and developed in nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) mixture. The purple-blue precipitates

were identified as positive signal.

In situ hybridization

A nonisotopic method, with biotin-labeled DNA probes to HPV16, 18, 31, 33 and 35 (Dako, Glostrup, Denmark), was used to determine the genotype of HPV in the American sections (6). Cells with dark purple precipitates in nuclei were considered positive for HPV infection.

Immunohistochemical staining and immunoblotting

Immunohistochemical staining was performed on paraffin-embedded tissues by an immunoperoxidase method as previously described (6,14-18). Following removal of paraffin with xylene and alcohol, pathological sections were incubated with antibodies specific to ATAD3A (18), hepatocyte growth factor (HGF), HGF receptor (HGFR, or product of proto-oncogene c-*met*, c-MET), IL-8 (R&D systems, Minneapolis, MN) or Ki-67 (Zymed, San Francisco, CA). The slides were then treated with peroxidase-conjugated secondary antibodies, and developed in 3-amino-9-ethylcarbazole (AEC). The crimson precipitates were identified as positive staining. The non-tumor counterpart of the cervical tissue was served as a negative control, and a section of mouse liver tissue was used as positive control for each run of immunohistochemical staining. The same antibodies were used for immunoblotting, of which the procedure has been described previously (14-18).

Cell cultures, and drug treatment

In this study, five human cell lines were used, including three squamous cell carcinomas (SKG-I, SKG-II and SKG-IIIa), and two endocervical adenocarcinomas (Nuz and HeLa). Culture media and fetal calf serum (FCS) were from Gibco Laboratories (Grand Island, NY). Other materials were reagent grade from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany). The cells were grown at 37°C in RPMI1640 plus 10% FCS, and all the media were supplemented with 3 mM glutamine, penicillin (100 IU/ml) and streptomycin (100

μg/ml).

Drug-sensitivity assay

Drug-sensitivity was measured by a WST-1 assay.20 Cells were seeded at 100, 1,000, and 5,000 cells/96-well plate 18 hours prior to drug challenge. Cells were continuously incubated with various concentrations (ranging from 0.1 to 50 μM) of anticancer drugs, e.g., cisplatin, doxorubicin and paclitaxel. The negative control group included cells that were treated with the same concentration of DMSO, a solvent for the drug. Total survival of the cells was determined 72 hrs following drug challenge, and percent survival was estimated by dividing optical absorbance resulted from each test group with that of control group. Each experiment was done in triplicates, and the optical absorbance was measured by coloration of oxidized substrate, WST-1 (BioVision, Mountain View, CA), which was catalyzed by mitochondrial dehydrogenase.

Inhibition of ATAD3A expression by siRNA-producing lentivirus

ATAD3A expression in SKG-I cells was inhibited by small interference RNA (siRNA) produced by lentivirus infection (18). The infected cells were selected with 1.0 μg/ml puromycin for 48 hours. ATAD3A expression in cells was examined by immunoblotting.

Slide evaluation

Non-tumor cervical tissue was served as internal negative control. Slides were evaluated by two independent pathologists without clinicopathological knowledge. An ImmunoReactive Scoring system was adapted for the study (21). Briefly, a specimen was considered positive if more than 25% of cancer cells were positively stained; or negative, if less than 25% positively stained.

Statistical analysis

Relations between ATAD3A expression and clinicopathological parameters were analyzed by Chi-Square test. Survival curves were plotted with a method of Kaplan-Meier

(22). Statistical difference of survivals between different groups was compared by the log rank test (23). Statistical analysis was performed using GraphPad Prism5 statistical software (San Diego, CA). Statistical significance was set at p value < 0.05.

Results

High-risk genotype HPV infection and ATAD3A expression

High-risk HPV (hrHPV), e.g., HPV16, 18, 31, 33, 52, 56, 58, signal was detected in 184 (88.9%) of 207 specimens, among which 158 (85.9%) specimens contained single genotype and 26 (14.1%) contained double genotypes. Low-risk HPV (lrHPV), e.g., HPV43, 54, 61, 70, was detected in 15 (7.25%) of 207 specimens. Eight patients were negative for HPV infection. Genotype distribution of hrHPV infection is listed in Table 1. Interestingly, ATAD3A (Figures 1A-1D), which was not expressed in non-tumor uterine cervical tissues (NTUCT), was detected in 159 (86.4%) of hrHPV^{+}, 3 (20%) of lrHPV^{+} and 2 (25%) of HPV^{-} cases. Among hrHPV^{-} cases (n = 23), only 5 (21.7%) expressed ATAD3A. ATAD3A expression was positively correlated with hrHPV infection [$p < 0.001$, and Spearman correlation (SC) = 0.501]. Cell cycle-related Ki-67 was detected in 64 (30.09%) of specimens. Pro-inflammatory or metastasis-related cytokines and receptor, such as, HGF, IL-8 and c-MET, were detected in 72 (34.8%), 135 (65.2%) and 122 (58.9%) of 207 samples. Correlation of ATAD3A with c-MET was positive ($p < 0.001$, SC = 0.299). So was that with IL-8 ($p = 0.03$, SC = 0.151) and Ki-67 ($p = 0.05$, SC = 0.136). Statistically significant difference, moreover, was found with respect to FIGO stages ($p = 0.014$, SC = -0.217), mitotic index ($p < 0.001$) and lymph node involvement ($p = 0.001$, SC = 0.225). No significant difference was found in tumor type or expression of HGF. Results are summarized in Table 2. ATAD3A overexpression was detected in 142 (69.3%) of 205 American specimens (Figures 1C & 1D). Using *in situ* hybridization (6), HPV signals were detected in 156 (76.09%) of the American specimens. Correlation of HPV infection with ATAD3A expression was positive as well ($p < 0.001$, SC = 0.767). A statistical difference was significant in ATAD3A expression between the American specimens and the Chinese UCC patients ($p = 0.021$). Tendency to detect overexpression of ATAD3A in Chinese patients was 1.69-fold higher than that in the American specimens.

Among the 164 $ATAD3A^{+}$ patients, 44 (26.8%) had tumor recurrence, and among the 43 $ATAD3A^{-}$ patients, 4 (9.3%) patients had tumor recurrence. The statistical difference of tumor recurrence was significant ($p = 0.008$). Survival rate of $ATAD3A^{+}$ patients was also significantly worse than that of $ATAD3A^{-}$ patients (Figure 2A, $p = 0.0016$). Using multivariate analysis, FIGO stages, hrHPV infection, lymph node involvement and ATAD3A overexpression were four important factors correlated with poor prognosis for UCC patients. When survival of UCC patients, of whom tumors were above stage Ib2 and received cisplatin-containing chemoradiotherapy, was analyzed according to ATAD3A expression, the difference was significant as well (Figure 2B, $p = 0.0467$), confirming that ATAD3A overexpression was correlated with drug resistance in UCC cells.

Association of ATAD3A expression and hrHPV infection with drug resistance in cancer cell lines derived from cervix

As determined by immunoblotting, protein levels of ATAD3A were evidently different among various uterine cervical cancer cell lines. Level of ATAD3A was high in SKG-I, SKG-IIIa, NUZ and HeLa, but low in SKG-II cells (Figure 3A). Among four HPV-positive squamous cell carcinoma cell lines, three had high levels of ATAD3A expression. Based on ATAD3A levels and cell types, we thereby chose SKG-I and SKG-II for a cytotoxicity study.

Our previous study showed that SKG-I was significantly more resistant to anticancer drugs than SKG-II, which we had attributed to the presence of DDH (6). However, DDH does not directly inactivate anticancer chemotherapeutics (14,15), suggesting that desensitization effect on anticancer drugs may involve other factors. To investigate the role of ATAD3A in drug resistance, we silenced ATAD3A ($ATD3A^{kd}$) expression using a siRNA method. As shown in Figure 3B, after treatment with siRNA-lentivirus, ATAD3A expression was decreased around 10-fold in SKG-I cells. Drug resistance to cisplatin, ifosfamide,

cyclophosphamide, cytarabine, gemcitabine, 5-fluorouracil, docetane, paclitaxel, epirubicine, vincristine and doxorubicine markedly decreased in these cells (Figure 3C). The difference was significant ($p < 0.001$) in each of the above comparison. Interestingly, sing specific shRNA to silence E6 and E7 (HPV E6/7^{kd}) expression reduced protein levels of ATAD3A and β-actin (Figure 3D) as well as increased cell apoptosis and cisplatin sensitivity (Figure 3E). Silencing of E6 and E7 was determined by RT-PCR.[24]

Interestingly, addition of resveratrol reduced ATAD3A expression (Figure 4A). Because silencing of ATAD3A induced apoptosis and autophagocytosis (19), we therefore used electron microscopy to examine the ultrastructural differences between resveratrol-treated and ATAD3A^{kd} cells. Compared to the control cells (Figure 4B1), numbers of autophagosome and cytoplasmic vesicles evidently increased in ATAD3A^{kd} (Figures 4B2 & 4B3), and in resveratrol-treated cells (Figure 4B4). So were mitochondria with defected outer membranes and crumpled matrices. These data corresponded well with our previous observations that ATAD3A increased drug resistance of cancer cells, possibly *via* maintaining the appropriate membrane flow and the mitochondrial integrity (18,25). The results suggested that resveratrol might inhibit the trafficking of membrane phospholipids and proteins. Interruption of the process could in some way initiate autophagy and the subsequent apoptosis.

Discussion

The anti-autophagic ability and facilitation of cancer development of ATAD3A have been shown to be *via* protection of mitochondrial integrity and prevention of cellular autophagy (18). In this study, we further demonstrated that ATAD3A expression was closely associated with high-risk HPV infection, validating our previous observations that hrHPV infection was not only critical for carcinogenesis, but also for the disease progression. The current results showed that using PCR and an Easychip HPV blot assay could simultaneously identify the types of HPV infection (6-8). However, using *in situ* hybridization could adequately locate the HPV infection in UCC, not in the neighboring tossues (6). Moreover, our results indicated that both HPV infection correlated with ATAD3A levels as well as those of c-MET, IL-8 and Ki67 as well as FIGO stages, lymph node involvement and patients' survival, which corresponded well with our prior studies that expression of these genes might be orchestrated with one another to form a malicious cycle in favor of cancer progression (6, 26,27).It is worth noting that, Chinese UCC patients had a higher tendency of overexpressing ATAD3A than the American patients. Interestingly, silencing of ATAD3A expression markedly increased SKG I cell sensitivity to cisplatin, ifosfamide, cyclophosphamide, cytarabine, gemcitabine, 5-fluorouracil, docetane, paclitaxel, epirubicine, vincristine and doxorubicine. Knockdown of HPV16 E6/E7 in SKGI cells, furthermore, reduced protein levels of ATAD3A and β-actin as well as cisplatin resistance, confirming that E6 and E7 of oncogenic HPV were up-stream regulators of survival-related gene expression in UCC cells (28-30). Taken together, our current data indicated that drug resistance in cancer cells, in addition to elevated nuclear DNA repair and cytoplasmic detoxification activities (14-16, 31), could be enforced by proteins, such as ATAD3A, which was essential for the maintenance of mitochondrial membrane integrity (18,19).

As noted above, ATAD3A is an ATPase which is involved in an alternative transport of

proteins and phospholipids from the endoplasmic reticulum (ER), mitochondria-associated membrane to the mitochondria (18). Silencing of the ATAD3A expression not only altered mitochondrial morphology, but also induced autophagy (18). However, serum starvation increased both protein and mRNA levels of ATAD3A in cancer cells, suggesting that a cell self-defense mechanism to conserve energy consumption, while to sustain cell survival was consummated to avoid a drastic initiation of autophagy (32,33). During initiation of autophagy, the C-terminal 5 amino acids are removed from the microtubule-associated protein light chain 3 (LC-3) to form a LC-3I, which is then conjugated to a phosphotidylethanolamine (PE) to form a LC-3II, the known marker of autophagy (34). However, the site for LC-3 conjugation to PE, as well as the formation of phagophores, the energy source for the aggregation of autophagosomes and the fusion of autophagosomes with lysosomes is yet to be characterized (32-34). Recently, Hailey et al. showed that conjugation of LC-3I to PE could be taken place on the mitochondria, where the phosphotidylserine (PS) was converted to PE, and where the prospective phagophore, a precursor of autophagosome, was formed during serum starvation (35).That human mitochondrial fission protein 1 (hFis 1) and mitofusin 2 (Mfn-2) were detected on both mitochondria and autophagosomes and that deletion of mitofusin 2 (*Mfn-2*) gene inhibited the formation of autophagosomes suggested that hFis1 was required for phagophore formation and that Mfn-2, in addition to mitochondrial fusion and tethering between the ER and the mitochondria (36), was required for the fusion of microphagosomes into autophagosomes.

By showing that hypoxia-induced autophagic vesicles were encircled by DRP1 and human homolog of yeast Rad23 protein A (hHR23A) as well, our previous data suggested that DRP1 and hHR23A might respectively play a role in mammalian cell autophagy,[17] based on the fact that hFis1 required DRP1 to carry out fission activities (37). Moreover, our current results provided a reasonable interpretation for the involvement of ATAD3A in autophagy and

apoptosis, because ATAD3A might be able to provide energy for the movement of transport vesicles, ferrying phospholipids and proteins from the ER, the mitochondria-associated membranes to the mitochondria. Inhibition of ATAD3A function would surely cause short supply of energy required for transporting phospholipids and proteins to mitochondria, and membrane abrasions of mitochondria. The loss of mitochondrial membrane integrity would then lead to a failure to keep programmed cell death-related proteins, e.g., apoptosis-inducing factor (AIF) and cytochrome c, in check within the mitochondria. This concept was strengthened by the observations that resveratrol inhibited ATAD3A expression and induced autophagy as well as apoptosis, which corresponded well with results reported by Hsu *et al* (38).

In conclusion, our current results showed that ATAD3A expression was frequently detected in high-risk HPV-positive UCC specimens and was correlated with FIGO stages, lymph node involvement and patients' survival. The HPV infection may stabilize ATAD3A expression to prevent initiation of cell autophagy and the subsequent cell apoptosis, hence to increase drug resistance in UCC cells.

Conflict of interest

The authors declare that they have no conflict of interest in this study.

Acknowledgments

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Figure legends

Figure 1. Representative examples of ATAD3A expression in the uterine cervical cancer cells. ATAD3A was detected by immunohistochemistry, and the slide was counterstained with hematoxylin. (A) In the uterine cervical cancer, overexpression of ATAD3A was detected in tumor nests (original magnification × 100). B, magnification of the Chinese UCC tissue showed that overexpression of ATAD3A was mainly detected in the center of tumor nest (arrow). (original magnification × 400); C, in a tissue microarray of American UCC specimens, overexpression of ATAD3A was detected in UCC cells (original magnification × 100). D, magnification of the American tissue microarray showed that overexpression of ATAD3A was mainly detected in the center of tumor nest (arrows). (original magnification × 400)

Figure 2. Cumulative survival curves in Chinese UCC patients. Survival curves were plotted with method of Kaplan and Meier. Statistical difference of survival between the two groups was compared by a log rank test. Patient groups were divided according to (A) expression of ATAD3A ($p = 0.0016$), (B) Cumulative survival curves of Chinese UCC patients in whom tumor was beyond stage Ib2 and received cisplatin-contained chemoradiotherapy. Patient groups were divided according to expression of ATAD3A ($p = 0.0467$).

Figure 3. Expression of ATAD3A in various cancer cell lines derived from cervix was detected by immunoblot analysis. (A) Immunoblot analysis of ATAD3A expressions in cancer cells derived from cervix. Expression of 66-kDa ATAD3A was detected in all 3 HPV-infected squamous cell cancer (SCC), though the expression levels varied. Expression of c-Met was detected in all 5 cell lines. Interestingly, expression of phosphorylated c-Met (upper band) correlated with that of ATAD3A. (B) SKG-I cells were infected by lentivirus or virus

containing ATAD3A-targeting siRNA-generating hairpin sequence (shATAD3A) to suppress ATAD3A expression. ATAD3A content was verified by quantitative RT-PCR (upper panel) and immunoblotting (lower panel). After cells infected with ATAD3A-targeting shRNA-lentivirus, ATAD3A expression was decreased about 10-fold in shATAD3A-infected SKG-I cells. (C) Drug sensitivity of SKG-I, vector virus- and shATAD3A-infected cells to anticancer drugs. Percent survival is plotted against a single dose of the drug. Survivals are the mean of triplicate experiments. SKG-I and vector virus-infected cells that had higher content of ATAD3A were more resistant to anticancer drugs. The difference was significant ($p < 0.001$). No significant difference was found between SKG-I and vector virus-infected cells ($p = 0.94$). □: SKG-I, ▒: SKG-I infected with vector virus, ▨: SKG-I infected with shATAD3A virus. (D) Silencing of E6 and E7 expression by shRNAs (HPV E6/7^{kd}) reduced expression of ATAD3A and β-actin as determined by immunoblotting. (E) Silencing of E6/E7 increased cisplatin sensitivity in UCC cells. ○, SKG I, wild-type; ●, SKG I, E6/$E7^{kd}$ with scrambled shRNA; Δ, SKG I, E6/$E7^{kd}$ with shRNA. F-test, $p < 0.01$

Figure 4. The effect of resveratrol on ATAD3A expression in SKG-I cells. (A) Resveratrol reduced ATAD3A expression in a dose-dependent fashion. (B) Effect of ATAD3A expression on ultrastructural changes. Compared to (B1) the wild-type cells, (B2) the evident appearance of autophagic vacuoles (black arrowheads) and degenerated mitochondria or silhouette of mitochondria (white arrow heads) were frequently detected in $ATAD3A^{kd}$ cells. Small vesicles in the cytoplasm became more evident, and, occasionally, peroxisome (black arrow) was identified in $ATAD3A^{kd}$ cells. (B3) In some cases, autophagosome (white arrow), containing degenerated mitochondria (black arrowheads), was observed. (B4) In resveratrol-treated cells, autophagic vacuoles (black arrowheads) and degenerated mitochondria as well as silhouette of mitochondria (white arrow heads) were frequently

detected. Small vesicles in the cytoplasm also become more evident. However, no peroxisome was identified in resveratrol-treated cells.