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Apoptotic resistance, cell cycle dysregulation and autophagy activation contribute to arsenic carcinogenesis

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Abstract

Background: Arsenic is a common environmental pollutant, chronic arsenic exposure causes multiple cancers including skin cancer. However, the underlying mechanisms of arsenic-driven skin carcinogenesis remains unclear.

Methods: HaCaT cells were exposed to a low level of arsenic (100nM) for 28 weeks. Then, xenograft mouse model and H&E staining were used to test their ability of tumors formation. The effect of arsenic on apoptosis demonstrated by TUNEL assay. XIAP and cIAP1 were determined by immunocytochemistry. MMP9, p16, Cyclin D1, CDK4 and Rb were detected by Real-time PCR. The protein expression level of Cyclin D1 was detected by Western blot . The formation of autophagosome was examined by a transmission electron microscope.

Results: After HaCaT cells were chronic exposed to arsenic for 28 weeks, malignant transformation occurred as evidenced by the formation of highly aggressive squamous cell carcinoma after inoculation into nude mice. In addition to increased secretion of MMP9, apoptotic resistance generalized and members of the inhibitor of apoptosis (IAP), including XIAP and cIAP1, were significantly elevated in arsenic-transformed cells (termed AS-TM). Furthermore, p16, Cyclin D1, CDK4, and Rb were significantly increased in AS-TM. The protein expression level of Cyclin D1 in AS-TM cells was significantly higher than that in control. More interesting, arsenic was found to induce autophagy after chronic arsenic exposure, heightened

autophagosome release was observed in AS-TM.

Conclusion: Apoptotic resistance, cell cycle dysregulation and activation of autophagy are the underlying mechanisms of arsenic-driven skin carcinogenesis, which provide new insight on the pathogenesis of arsenic-induced skin cancer.

Keywords: Arsenic, carcinogenesis, apoptotic resistance , cell cycle, autophagy

Background

Arsenicosis is a serious and widespread global public health problem[1,2] with more than 200 million people at risk of toxic arsenic exposure from groundwater and food contamination[3-5]. Chronic exposure to Arsenic (As) has been correlated with cancers of the skin, lung, liver, bladder, and kidneys as well as noncarcinogenic diseases such as skin lesions, cardiovascular disease, reproductive defects, neurological injuries, and diabetes mellitus[6]. At present, the toxic effects of arsenic exposure on skin have been documented. Arsenic was demonstrated that the exposure of arsenic in humans is associated with a marked increase in skin cancer, such as squamous cell carcinoma and basal cell carcinoma[7,8]. Studies have shown that human keratinocytes (HaCaT) can be converted into acquired malignant phenotype cells by continuous exposure to low concentrations of arsenic, which was evidenced by the formation of highly aggressive squamous cell carcinoma after inoculation into nude mice[9]. It has been recognized as a cell research model for arsenic-induced skin cancer. Due to the complex role of arsenic, although scholars have proposed some hypothesis mechanisms of arsenic carcinogenesis, the exact mechanism of arsenic

carcinogenesis is still unclear.

Apoptosis, a closely regulated programmed cell death mechanism, is an essential process to maintain tissue homeostasis. Resistance to apoptotic stimuli is one of the hallmarks of cancer[10], and can be achieved by overexpression of anti-apoptotic proteins. There are several factors have been found correlated to the carcinoma cell apoptotic resistance, including members of the inhibitor of apoptosis (IAP). X-linked inhibitor of apoptosis protein(XIAP) and cellular inhibitor of apoptosis protein 1(cIAP1) were the most potent caspase inhibitor in the IAP family, they play a critical role in the anti-apoptotic and pro-survival signaling pathways. XIAP and cIAP1 are highly expressed in almost all of a series of 60 human cancer cell lines studied[11]. It remains unclear how IAPs regulate the cell cycle. The typical example is survivin, an IAP that connects antiapoptotic pathways and the cell cycle[12]. XIAP and its E3 ligase also played a crucial role in regulation of Cyclin D1 expression in cancer cells[13]. Cell cycle dysregulation is an important mechanism of tumorigenesis, the abnormal changes of the regulatory factors in the G1/S phase of the cell cycle are particularly closely related to cell carcinogenesis. The role and significance of the p16-cyclin D1/CDK4-pRb pathway in various tumors has been confirmed[14]. In recent years, Eshkoor et al[14] found that Cyclin D1, P16 are highly expressed in squamous cell carcinoma of the skin.

Autophagy is considered as an evolutionarily cell survival process, which is responsible for degradation of long-lived proteins and removal of dysfunctional organelles[15]. It seems that autophagy and apoptosis function appositely. While

apoptosis is a cellular suicidal program, autophagy can either be a cell survival mechanism as a homeostatic process or a stress-induced cell death pathway depending on variable context [16,17]. High levels of XIAP and cIAP1 expression can induce the formation of autophagosomes by up-regulating Beclin 1 expression via the activation of the NF κ B pathway[18]. Cyclin D1 activity can regulate autophagy and senescence in the mammary epithelium[19].

Although apoptosis, cell cycle and autophagy have been well documented, the role of apoptosis, cell cycle and autophagy in arsenic carcinogenesis and the interaction between them have not been well understood in human Keratinocytes. Therefore, in search for crosstalk between apoptosis, cell cycle and autophagy pathways and to see how arsenic affects these processes, will help to better understand the mechanism of arsenic carcinogenesis and find new targets for clinical prevention and treatment of skin cancer.

Methods

Cell culture and arsenic exposure

The human keratinocyte cell line HaCaT (maintained in our lab) cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal bovine serum), 100 U of penicillin/ml, and 100 mg of streptomycin/ml. Cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere. For chronic arsenic exposure, cells were maintained continuously in medium containing 100 nM of arsenic trioxide (Sigma, St. Louis, MO) for 28 weeks[20,21]. All the materials for

cell culture were purchased from Thermo Scientific HyClone (Logan, UT, USA).

Xenograft mouse model

To test their ability to form tumors, female BALB/c nude mice (4-6 weeks old) were randomly divided into 2 groups: those that received 1) passage-matched control HaCaT (n=5), 2) AS-TM (n=15). Cells (2×10^6 cells/0.2 mL) were suspended in cold PBS and subcutaneously injected into the left armpit of mice. The animal study was approved by the Ethics Committee of The First Affiliated Hospital (Dalian Medical University, China). The tumor volumes were calculated using the formula: length \times width \times height \times 0.52. Mice were monitored until tumors reached 1.0 cm³ total volume, at which time mice were euthanized, tumors were extracted and embedded in paraffin, sectioned, stained with H&E, and analyzed by light microscopy.

H&E staining

After the tissues are fixed in neutral formalin, embedded in paraffin and sliced. 4 μ m thick sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS. And then stained with hematoxylin and eosin (H&E). After staining, sections were dehydrated through increasing concentrations of ethanol and xylene. Observing the pathological changes of tumorigenic tissues under light microscope.

TUNEL assay

For the terminal deoxynucleotidyltransferase 2'-Deoxyuridine, 5'-Triphosphate (dUTP)-nick end labeling (TUNEL) assay, HaCaT cells and AS-TM cells were fixed with 4% paraformaldehyde solution at room temperature for 30 min and permeabilized with 0.1% Triton X-100 for 2 min. Following washing with PBS, cells were incubated with TUNEL reagent (Beyotime Biotechnology, China). Incubate at 37 °C in the dark for 60 minutes. After PBS washing cells were also counterstained with 0.1 μg/mL DAPI for 5 min. Cells were analyzed under a fluorescence microscope.

Immunocytochemistry (ICC)

The cell suspension of each cell line was gently placed on Poly-L-Lysine coated (PLL) coverslips (Neuvitro Corporation) in the bottom of each well of 12-well culture plates. The cells were then incubated at 37 °C in 5% CO₂. When cells on the coverslips reached 80% confluence, the media in the wells were removed, and cells on the coverslips were washed with PBS 3 times, fixed with 4% formalin for 30 min and washed with PBS 3 times again. Incubate for 15 minutes in 3% hydrogen peroxide and washed with PBS 3 times again. To perform ICC, the cells on the coverslips were incubated with 0.1% Triton, and washed with PBS 3 times. After blocking the goat serum for 30 minutes, incubate the XIAP (Abcam, ab28151) and cIAP1 antibody (Abcam, ab25939) for 12 h. After incubated secondary antibody and PBS washing, DAB color development for 5 minutes. Finally, the cells on each PLL coverslip were counterstained with hematoxylin and face-down mounted on a glass

slide. Under light microscope, the extent and subcellular localization of each XIAP and cIAP1 were observed.

Quantitative real-time PCR analysis

Total RNA was isolated by using TRIzol (Invitrogen,USA), and then cDNA was synthesized for each sample using the PrimeScript™ RT reagent Kit with gDNA Eraser following manufacturer's instructions (TaKaRa,Beijing,China). The SYBR Green PCR Kit (TaKaRa,Beijing,China) was used for quantitative real-time RT-PCR analysis. The primers were designed and synthesized by Sangon Biotech (Shanghai,China). β -actin was used as reference gene. Real time fluorescence detection was carried out using a MyiQ™ SingleColor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The primers used were listed as follows.

mRNA	Sequence
Cyclin D1	F: TGATGCTGGGCACTTCATCTG; R: TCCAATCATCCCGAATGAGAGTC
Rb	F: TTCAGCAGAACTGGCAGAAATG ; R: CAGTGTCACCAAGGTCCTGAG
p16	F: GGCACCAGAGGCAGTAACCA ; R: GGACCTTCGGTGACTGATGATCTAA
CDK4	F: CTTCTGCAGTCCACATATGCAACA; R: CAACTGGTCGGCTTCAGAGTTTC
MMP9	F: GACAAGCTCTTCGGCTTCTG; R: TCGCTGGTACAGGTCGAGTA
β -Actin	F: GCCCTAGACTTCGAGCAAGA; R: AGGAAGGAAGGCTGGAAGAG

Western blotting

Western blotting was performed as described previously[20]. Cells were lysed in RIPA buffer, and protein concentrations were determined by a BCA Protein Assay Kit (Santa Cruz, CA, USA). Cyclin D1 antibody (Santa Cruz, sc-4074) was diluted at 1:500. Blots were normalized to β -actin (Cell Signaling Technology, 3700S). Quantification of bands was performed using the ImageJ software (v4.18).

Transmission electron microscopy (TEM)

HaCaT and AS-TM cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate for 2h, postfixed with 1% OsO₄ for 2h and washed in 0.1 M sodium cacodylate 3 times, 15 min each. Then, samples were dehydrated with graded alcohol (50%, 70%, 90%, 90% ethanol+90% acetone, 90% acetone,100% acetone; 15 min each). After embedded the samples in low viscosity resin, ultrathin sections were cut by Ultramicrotome (EMUC6, Leica, Germany), and then counterstained with 3% uranyl acetate and lead citrate[22]. The formation of autophagosome was examined by a transmission electron microscope (JEM1230, JEOL, Japan).

Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (SPSS Inc.,Chicago,IL, USA). Data were expressed as mean \pm SD. Student' s t test was used to determine the statistical differences. P<0.05 was considered statistically significant.

Results

Low dosage of arsenic induces HaCaT cells malignant transformation

To achieve oncogenic transformation, HaCaT cells were continuously exposed to a low level (100 nM) of arsenic trioxide for 28 weeks. Our previous research[20] has confirmed that after 28 weeks of continuous arsenic exposure, the arsenic-treated cells (AS-TM) exhibited unique morphological alterations with the frequent occurrence of giant multinuclear cells, which are common during malignant transformation and in tumors. The AS-TM cells proliferation and anchorage-independent growth were significantly promoted after malignant transformation[20]. Here, Malignant transformation was confirmed when the AS-TM cells produced aggressive cancers after inoculation into nude mice (Fig.1A). 93.33% (14/15) mice inoculated with the AS-TM cells developed tumors compared to 0% (0/5) mice inoculated with the control HaCaT cells. Histologic examination revealed the tumors to be highly aggressive squamous cell carcinoma (SCC) (Fig.1B). In addition, a marked increase in secretion of matrix metalloproteinase-9 (MMP9) from AS-TM cells occurred (Fig. 1C). Extracellular MMP9 activity was 11.5-fold higher in AS-TM cells compared to control. Thus, based on variety of criteria, it is clear that the AS-TM cells have acquired a highly malignant phenotype.

Arsenic exposure causes cell apoptotic resistance

To demonstrate the effect of arsenic on apoptosis, a TUNEL assay was applied. we found that there were some cells with positive signals in the HaCaT control group.

However, after exposure to arsenic (100 nM) for 28 weeks, numbers of cells with positive signals obviously decreased (Fig.2A-B). This indicated that arsenic promoted cell proliferation through apoptosis resistance.

Arsenic treatment induces high expression of anti-apoptotic proteins

To dissect arsenic's action mechanism, we further tested the expression level of XIAP and cIAP1 by immunocytochemistry. The results showed that both two anti-apoptotic proteins (XIAP and cIAP1) were highly expressed in AS-TM cells, of which XIAP was mainly expressed in cytoplasm and cIAP1 was mainly expressed in cytoplasm and nucleus (Fig.3A). The positive expression rates of XIAP and cIAP1 in AS-TM cells were significantly higher than those in HaCaT control cells(Fig.3B). These results indicated that arsenic may induce apoptotic resistance through anti-apoptotic proteins expression.

Arsenic treatment causes cell cycle dysregulation through upregulation of p16,Rb,CDK4 and Cyclin D1

Since XIAP played a crucial role in regulation of cyclin D1 expression in cancer cells[13]. Overexpression of cIAP1 increased the phosphorylation of Rb[23]. Cyclin D1, p16 are highly expressed in squamous cell carcinoma of the skin[14]. We suspect that arsenic treatment causes cell cycle dysregulation. Consistent with the assumption, Real-time PCR results shown that compared with the control HaCaT cells (0 weeks), the mRNA expression levels of p16, Rb, CDK4 and Cyclin D1 in the arsenic

treatment group at 6 weeks, 15 weeks and 28 weeks were significantly increased. The highest mRNA expression levels of P16, Rb and Cyclin D1 appeared at 6 weeks, and fell at 15 weeks and 28 weeks, but they were still significantly higher than the control group (Fig.4A). Cyclin D1, a key cell cycle regulator, is required for completion of the G₁/S transition in normal mammalian cells. It has been verified that high expression of Cyclin D1 is associated with invasion and metastasis of squamous cell carcinoma[24]. To confirm the biological significance of Cyclin D1 in arsenic carcinogenesis, the expression of Cyclin D1 was detected by Western Blot after 28 weeks of arsenic treatment . As shown in Fig. 4B, the expression level of Cyclin D1 in AS-TM cells was significantly higher than that in control. These results suggest that cell cycle regulation of the p16-cyclin D1/CDK4-pRb pathway is abnormal during arsenic-induced human skin cancer and the alteration of mRNA levels in the pathway is an early molecular event. Cyclin D1 is highly expressed during the malignant transformation of HaCaT cells and plays an important role in the process of arsenic-induced HaCaT malignant transformation.

Arsenic activated autophagy in HaCaT cells

Given high levels of XIAP and cIAP1 expression induce the formation of autophagosomes[18], the central role of Cyclin D1 as an integrator of growth-promoting signals that can also have an impact on autophagy[19]. We speculate that autophagy may play a role in arsenic carcinogenesis. To test this hypothesis, we studied AS-TM cells at the ultrastructural level using transmission

electron microscopy (TEM). As shown in Fig.5A, many autophagosomes, which are intracellular double-membraned vesicles characteristic of autophagy, were identified in AS-TM cells. The number of autophagosomes in the AS-TM group was significantly higher than control (Fig.5B).

Discussion

Arsenic is a common environmental pollutant, which has been studied for its toxic effects for many years. It has been well documented that chronic low-dose arsenic exposure increases proliferation and transformation of human and rodent cells[25]. However, the underlying mechanism of arsenic carcinogenicity is poorly understood. This study aimed to investigate a possible interaction between apoptosis, cell cycle, and autophagy and how the interaction relates to arsenic carcinogenesis. After HaCaT cells were continuously exposed to environmentally relevant levels of arsenic for a protracted period, malignant transformation occurred as evidenced by the formation of highly aggressive SCC (Fig.1A-B), a common form of skin cancer in arsenic-exposed humans[26], after inoculation of nude mice. Importantly, by studying this cell model system the important biomarker MMP9 for arsenic-induced malignant transformation was increased (Fig.1C). MMPs are a family of matrix-degrading enzymes that play a critical role in invasion and metastasis of malignant tumors and hypersecretion of MMPs is common in highly aggressive tumors[27]. Elevated expression levels of MMP9 are strongly correlated with malignant phenotype in SCC[28] and are characteristic of malignant transformation of cells[29]. Thus, the

dramatically increased MMP9 observed in AS-TM cells might be an important biomarker for arsenic-induced malignant transformation in human keratinocytes.

Resistance to apoptotic stimuli is a hallmark feature of various cancers. One of the mechanisms through which tumor cells are believed to acquire resistance to apoptosis is by overexpression of inhibitor of apoptosis proteins(IAPs)[30]. XIAP and cIAP1 were the most common members of IAP, XIAP is one of the best characterized member of the IAP family in terms of its potent caspase inhibitory mechanisms and is considered as the prototype of the IAP protein family [31]. cIAP1 can directly inhibit the activity of caspase-3. It has been reported that high levels of XIAP and cIAP1 expression could induce chemo-resistance and radio-resistance of human cancers[32,33]. Thus, XIAP and cIAP1 have been postulated to contribute to the development of some tumors[34]. Our study shown that both two anti-apoptotic proteins (XIAP and cIAP1) were highly expressed in AS-TM cells (Fig.3),which may contribute to the arsenic-induced malignant transformation of HaCaT cells and the apoptotic resistance (Fig.2).

Cell cycle dysregulation is an important mechanism of tumorigenesis. During tumor progression, changes in cell damage are achieved by regulating genes involved in the G1-S phase pathway in the cell cycle[35]. The regulation involves two pathways: one is the p16-cyclin D1/CDK4-pRb pathway and the other is the p53 pathway. Studies have confirmed that the p16-cyclin D1/CDK4-pRb pathway has been shown to be abnormal in many human cancers [36]. Cyclin D1 belongs to one of the G1 phase cyclins. Cyclin D1 binds to CDK4 and CDK6, phosphorylates the

downstream protein retinoblastoma protein (Rb), which releases the transcription factor E2F, promotes transcription of genes involved in DNA replication, promotes cell transition from G1 to S phase, and lead to cell proliferation[37-40]. Therefore, Cyclin D1 is an important family member of cyclins involved in cell cycle regulation, and its high expression is also thought to promote cell proliferation and promote tumor progression[41]. Eshkoo et al[14] showed that cyclin D1, p16 expression increased in squamous cell carcinoma tissues. In our study, in addition to the significant increase in XIAP expression long-term treatment of HaCaT cells with arsenic significantly increased cyclin D1 mRNA and protein expression levels (Fig.4 A-B). The expression of CyclinD1, a gene related to cell cycle regulation, was changed during the formation of HaCaT malignant phenotype cells induced by arsenic. The study also found that the level of Cyclin D1 mRNA expression in HaCaT cells at 28 weeks was not the highest. The highest value occurred at 6 weeks of the four time points, followed by a decrease in Cyclin D1 mRNA expression levels. The expression level of Cyclin D1 mRNA was highly expressed in the early stage of low-concentration arsenic treatment of HaCaT cells, suggesting that cyclin D1 gene may be an early molecular event in the formation of HaCaT malignant phenotype cells induced by arsenic. It provides important clues to our understanding of the early mechanisms of skin cancer formation.

Autophagy is a key mechanism for cancer cells adapted to a strict environment, which is involved in cancer progression and drug resistance[42]. Apoptosis, cell cycle and autophagy are interconnected cellular processes sharing regulatory components.

Anti-apoptotic protein XIAP played a crucial role in regulation of cyclin D1 expression in cancer cells[13]. Overexpression of cIAP1 increased the phosphorylation of Rb[23]. High levels of XIAP and cIAP1 expression induce the formation of autophagosomes[18], and the central role of cyclin D1 as an integrator of growth-promoting signals that can also have an impact on autophagy[19]. Therefore, the crosstalk between these pathways plays an important role in regulating physiological and pathophysiological processes. Our results indicated increases in autophagosomes caused by arsenic exposure in HaCaT cells (Fig.5 A-B), which suggest the enhancement of autophagy activity under the action of arsenic exposure.

Conclusion

In conclusion, the data from present study pointed out that arsenic-driven skin carcinogenesis involving intricate interactions between autophagy, cell cycle and apoptosis. While a low dose of arsenic can activate both processes, arsenic induce apoptotic resistance through anti-apoptotic proteins XIAP and cIAP1 expression may be an initial event in HaCaT cells and then cause cell cycle dysregulation and autophagy activation, leading to tumorigenesis (Fig.6). Therefore, apoptotic resistance, cell cycle dysregulation and autophagy activation may contribute to arsenic-induced carcinogenesis in the skin.

Abbreviations

HaCaT: Human keratinocyte cell line; MMP9: Matrix metalloproteinase-9; IAP:

Inhibitor of apoptosis; XIAP: X-linked inhibitor of apoptosis protein; cIAP1: Cellular inhibitor of apoptosis protein 1; AS-TM: Arsenic-transformed cells; ICC: Immunocytochemistry ; DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal bovine serum; H&E: Hematoxylin and eosin; TUNEL: deoxynucleotidyltransferase2'-Deoxyuridine,5'-Triphosphate (dUTP)-nick end labeling; PLLC: Poly-L-Lysing coated; TEM: Transmission electron microscopy; SCC: Squamous cell carcinoma; Rb: Retinoblastoma protein.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MYG designed and supervised the present study. YYZ, XLW and YHL performed the majority of the experiments. YYZ written the manuscript. WMZ analyzed the data.

YFW and GDP provided technical and material support. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Fig.1 Low dosage of arsenic induces HaCaT cells malignant transformation

(A) Arsenic exposure promotes tumor growth *in vivo*. AS-TM cells (2×10^6 cells/0.2 mL) were xenografted into nude mice. (B) Histologic examination of tumors formed by AS-TM cells after inoculation into the left armpit of nude mice. Tumors were fixed in formalin, embedded in paraffin, sectioned, and stained with H&E ($\times 200$). The staining pattern revealed the tumors to be aggressive squamous cell carcinoma. (C) Analysis of MMP9 mRNA expression level from AS-TM and passage-matched control cells. Changes in MMP9 mRNA expression were quantified by Real-time PCR. $n = 3$. * $p < 0.05$ vs. control cells.

Fig.2 Arsenic exposure causes cell apoptotic resistance

(A) Representative fluorescent figure of the apoptotic assay ($\times 200$). Blue DAPI was used to stain nuclei, and TdT tagged with a green fluorochrome was used to detect apoptotic DNA fragmentation. (B) The numbers of cells with positive signals obviously decreased in AS-TM group, compared with the control, the difference was statistically significant, *** $p < 0.001$.

Fig.3 Arsenic treatment induces high expression of anti-apoptotic proteins

(A) Protein levels of anti-apoptotic proteins (XIAP and cIAP1) in arsenic-transformed cells (AS-TM) and HaCaT cells were detected by Immunocytochemistry (ICC) ($\times 400$). (B) The positive expression rates of XIAP and cIAP1 in AS-TM cells were significantly higher than those in HaCaT control cells, data are presented as the mean \pm SD of at least three independent experiments, *** $p < 0.001$.

Fig.4 Arsenic treatment causes cell cycle dysregulation through upregulation of p16,Rb,CDK4 and Cyclin D1

(A) After arsenic treatment of HaCaT cells at different times, p16,Rb,CDK4 and Cyclin D1 mRNA expression levels were determined using Real-time PCR. $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. control cells (0 weeks). (B) Western Blot was applied to detect the Cyclin D1 protein expression levels after 28 weeks of arsenic treatment.

Fig.5 Arsenic activated autophagy in HaCaT cells

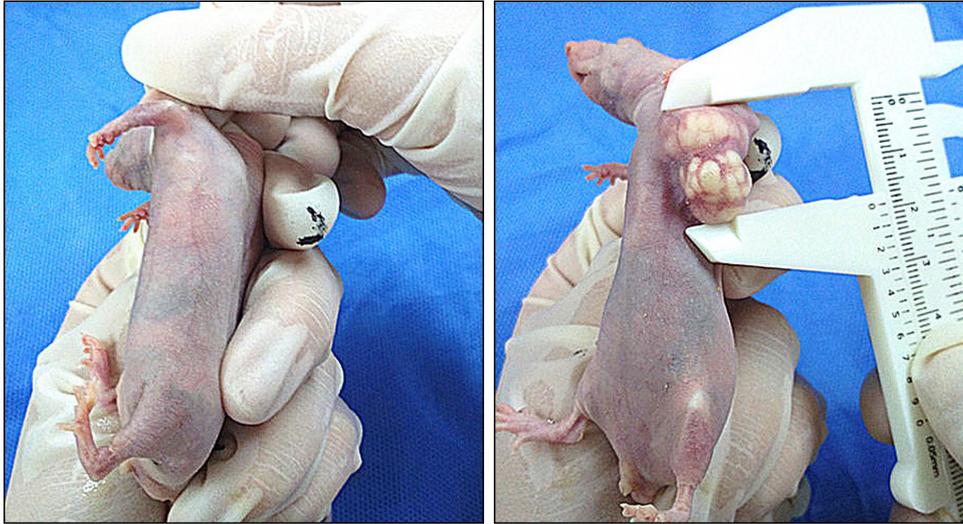
(A) TEM detected numerous double-membraned autophagosome-like vesicles in the AS-TM. (B) Autophagosomes were counted from image by TEM ($n=3$), *** $p < 0.001$. vs. HaCaT.

Fig.6 The schematic diagram elucidates the mechanism whereby apoptotic resistance, cell cycle dysregulation and autophagy activation contribute to arsenic

carcinogenesis. A low dose of arsenic treatment induce apoptotic resistance by increasing anti-apoptotic proteins XIAP and cIAP1 expression, XIAP and cIAP1 activate autophagy by regulating the p16-cyclin D1/CDK4-pRb pathway. At the same time, XIAP and cIAP1 can also directly activate autophagy, both of them ultimately leading to tumorigenesis.

Figure 1 A-C.

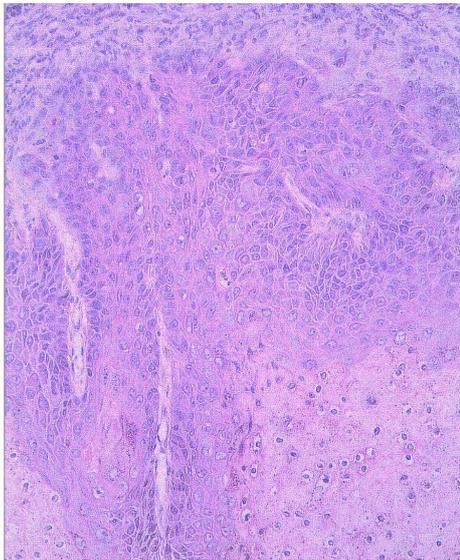
A



HaCaT

AS-TM

B



C

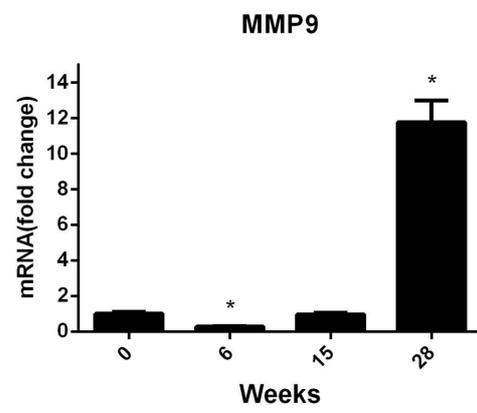


Figure 2 A-B.

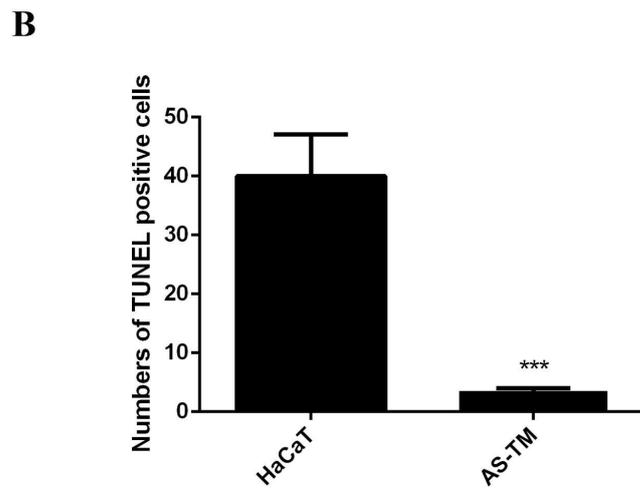
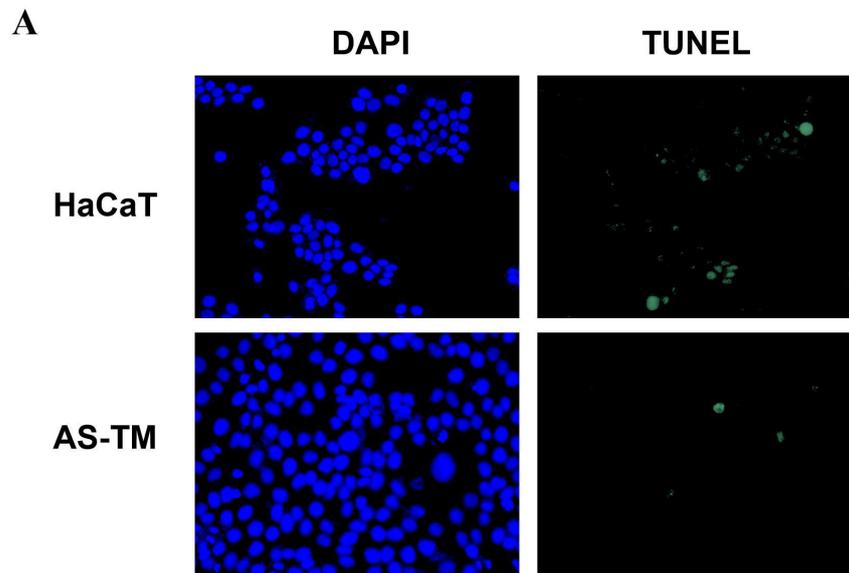
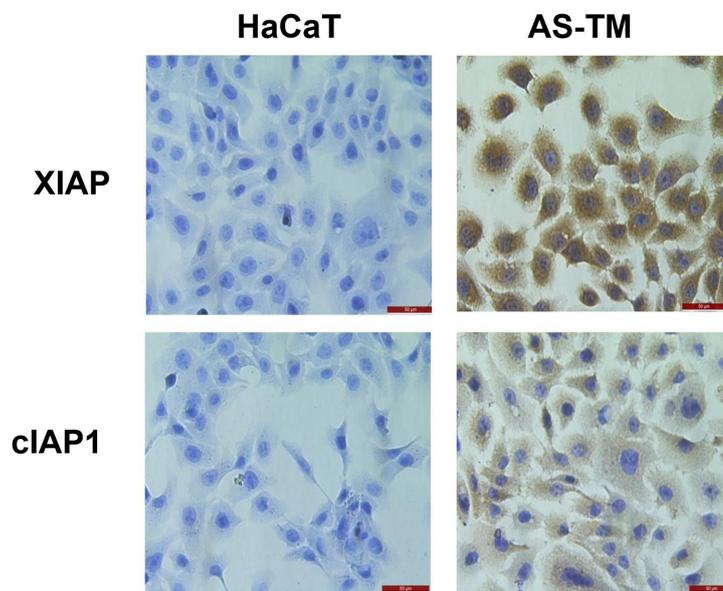


Figure 3 A-B.

A



B

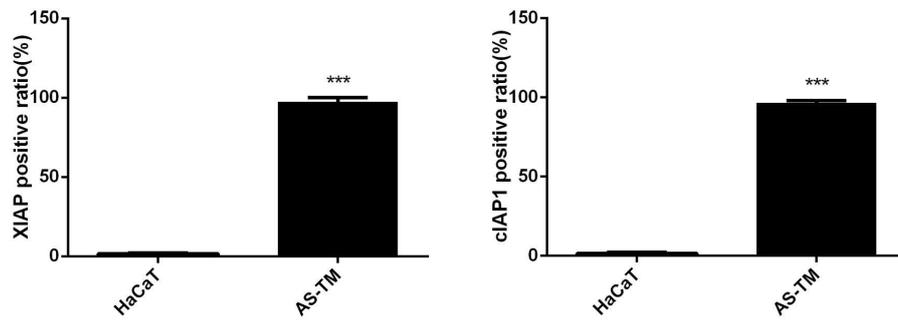
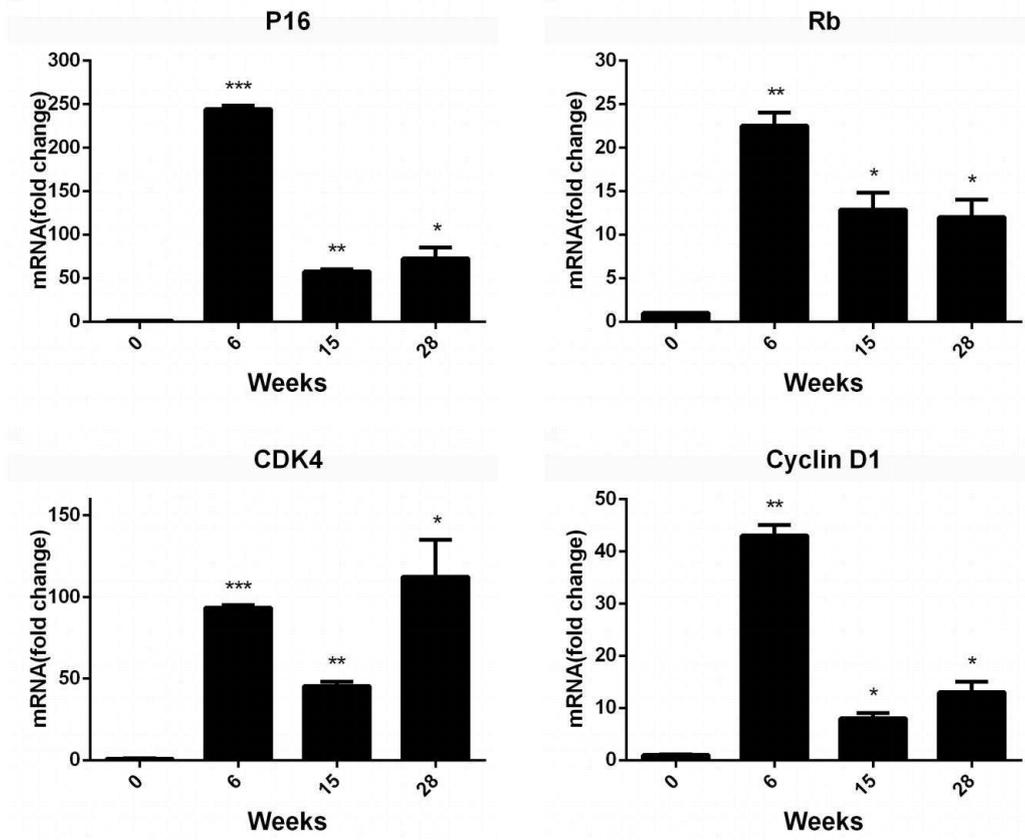


Figure 4 A-B.

A



B

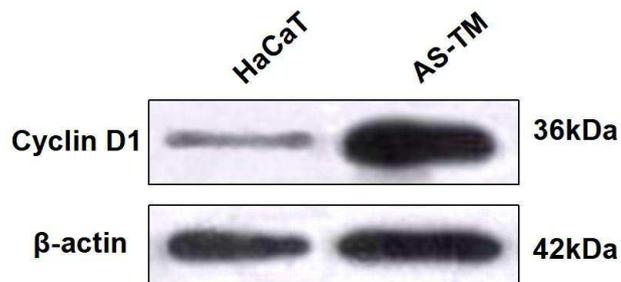
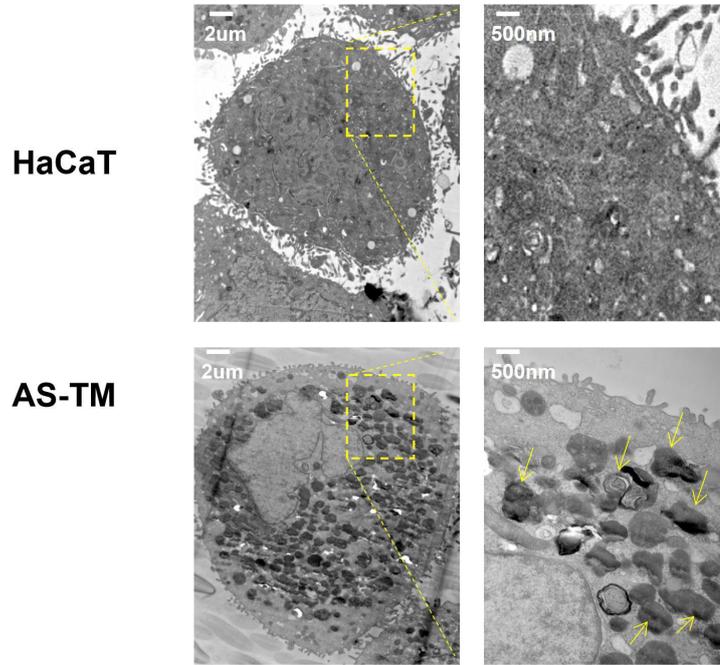


Figure 5 A-B.

A



B

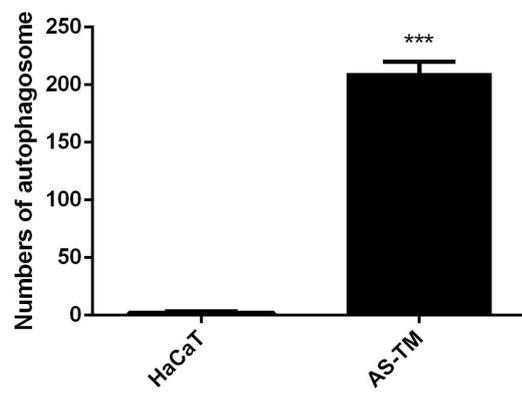
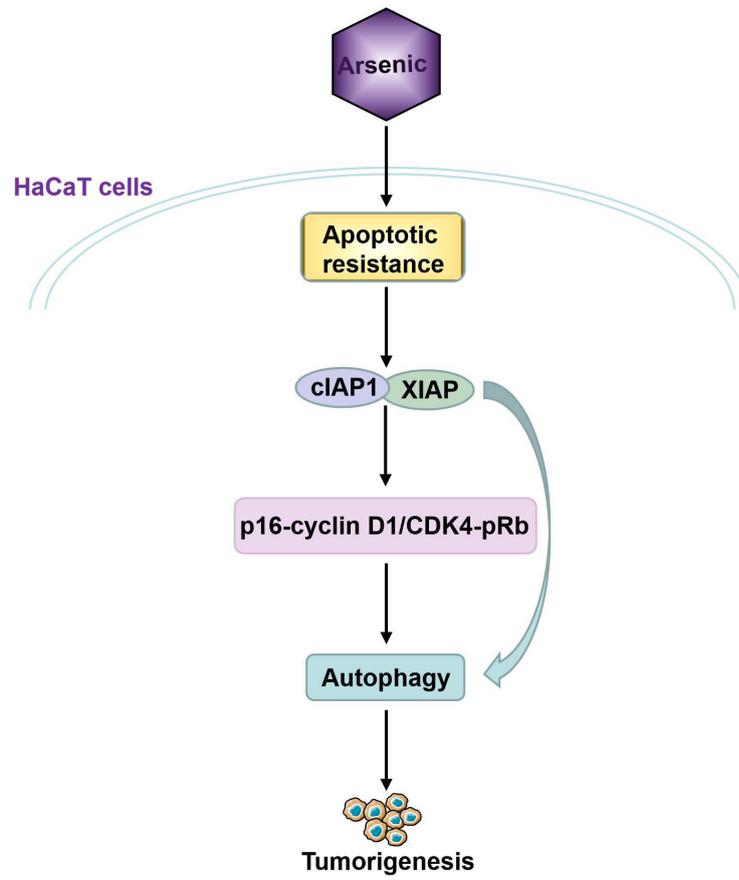
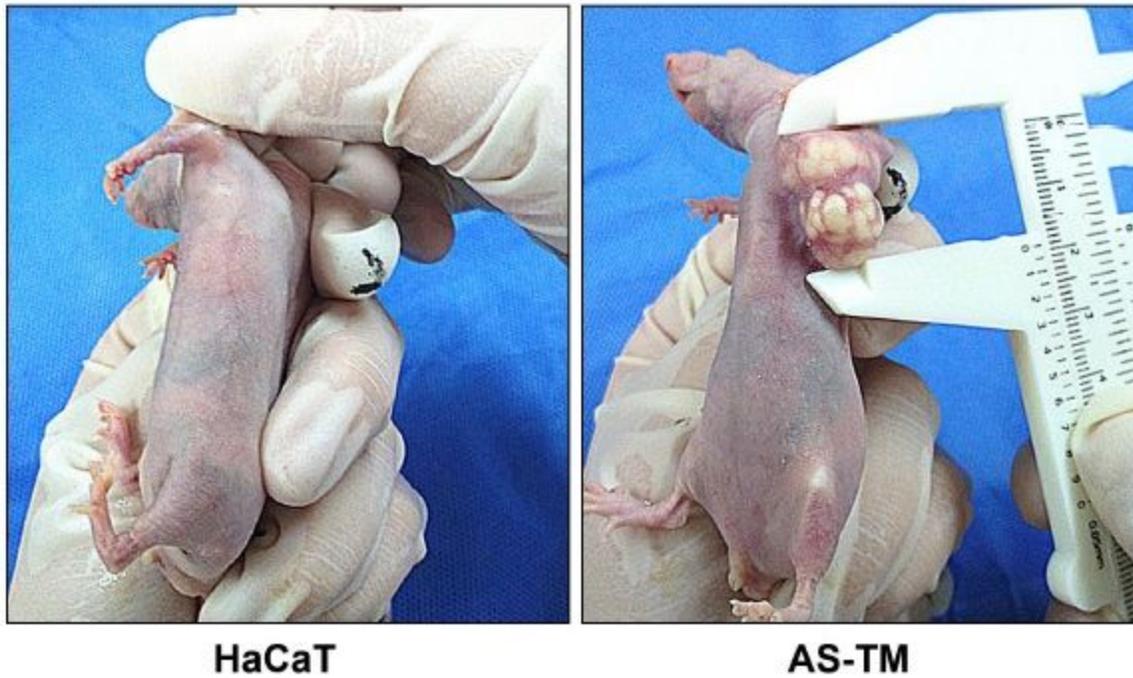


Figure 6 .

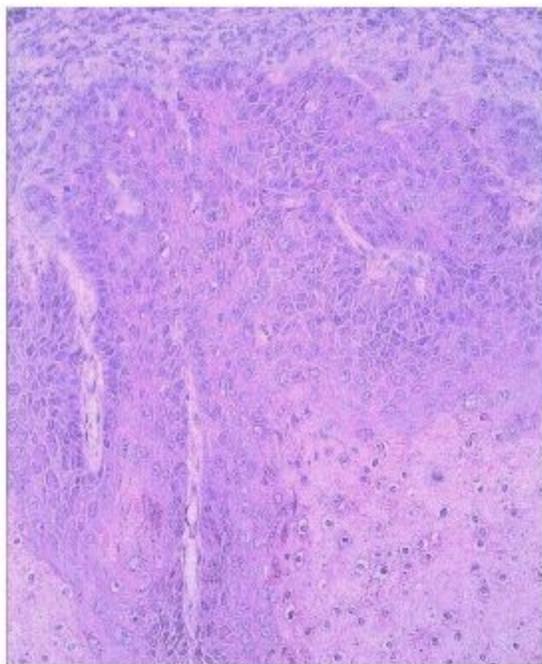


Figures

A



B



C

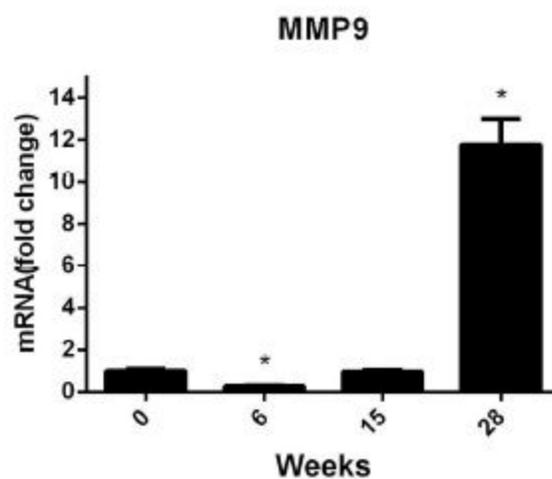


Figure 1

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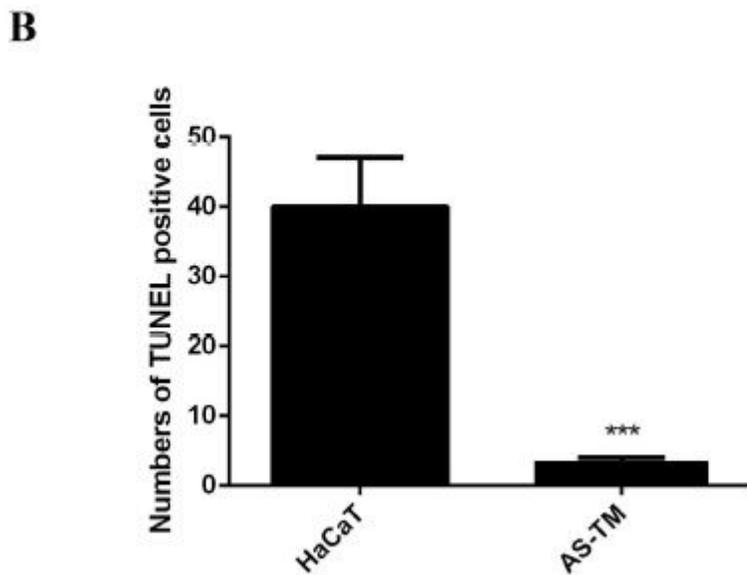
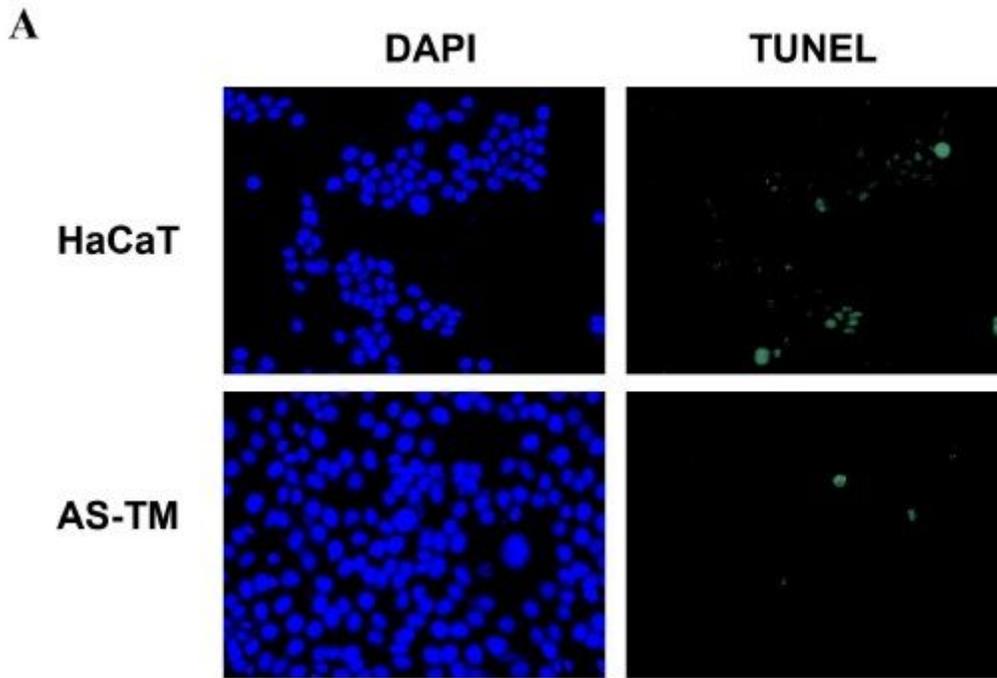


Figure 2

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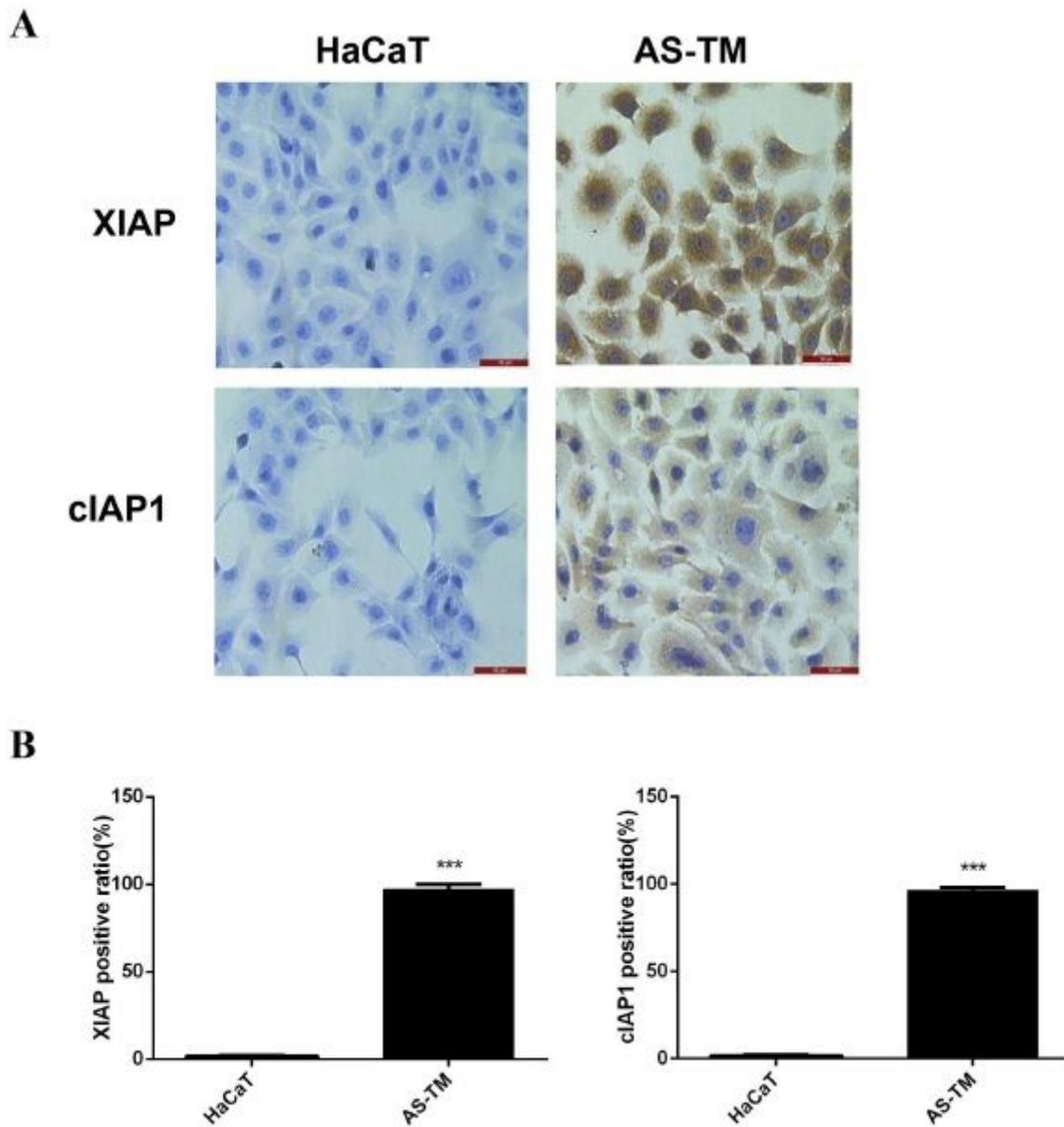


Figure 3

Arsenic treatment induces high expression of anti-apoptotic proteins (A) Protein levels of anti-apoptotic proteins (XIAP and cIAP1) in arsenic-transformed cells (AS-TM) and HaCaT cells were detected by Immunocytochemistry (ICC)($\times 400$). (B) The positive expression rates of XIAP and cIAP1 in AS-TM cells were significantly higher than those in HaCaT control cells, data are presented as the mean \pm SD of at least three independent experiments, *** $p < 0.001$.

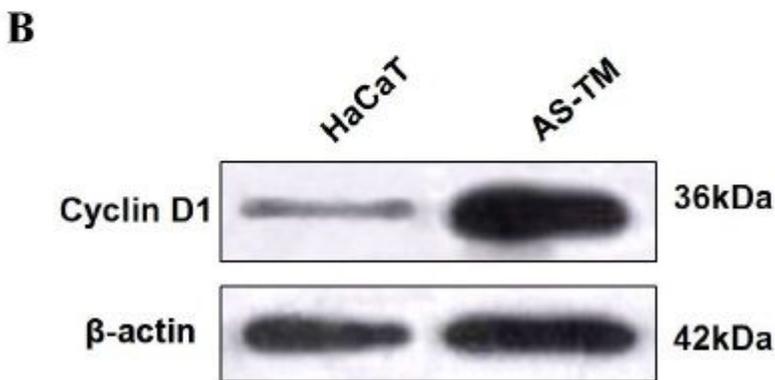
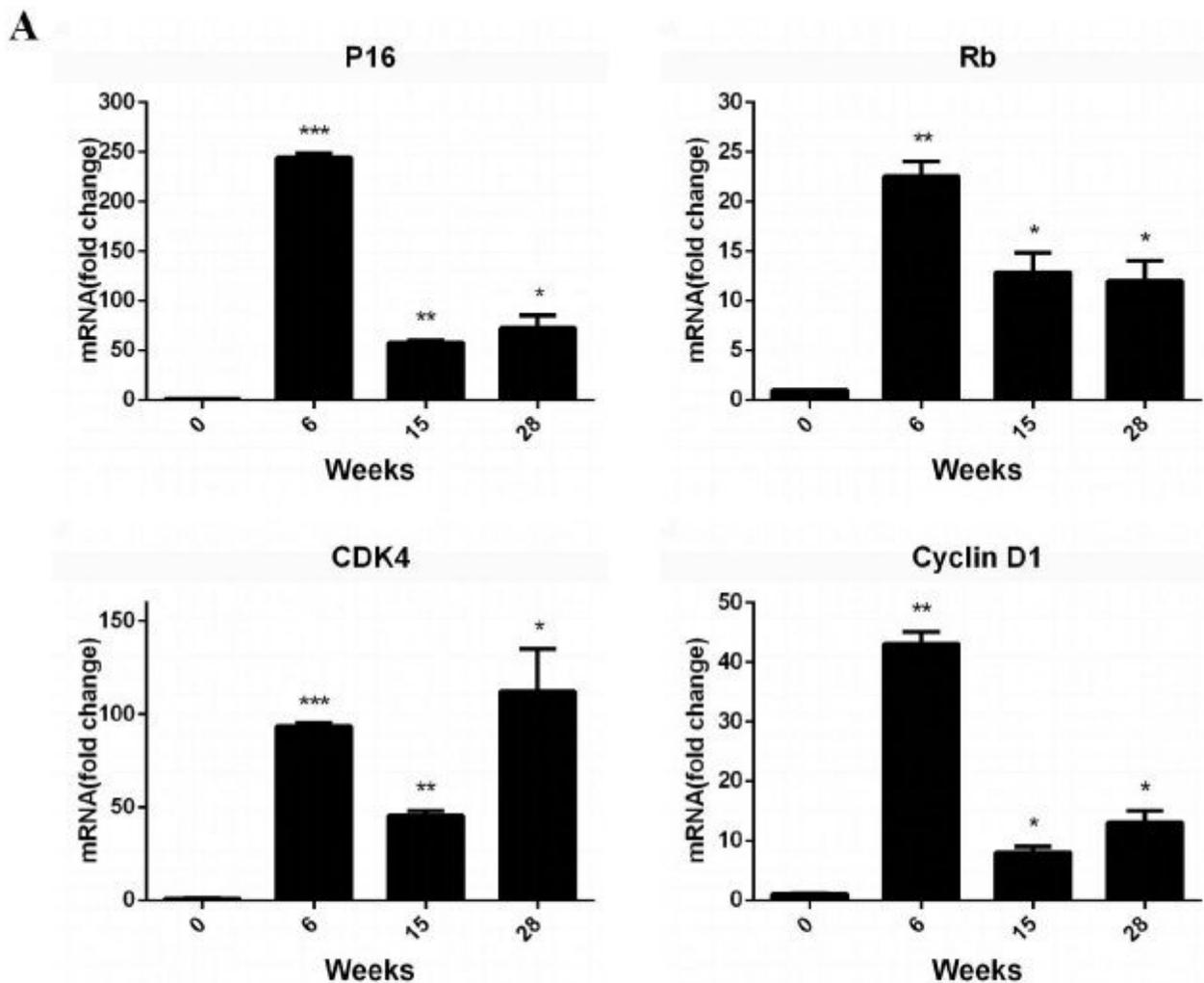
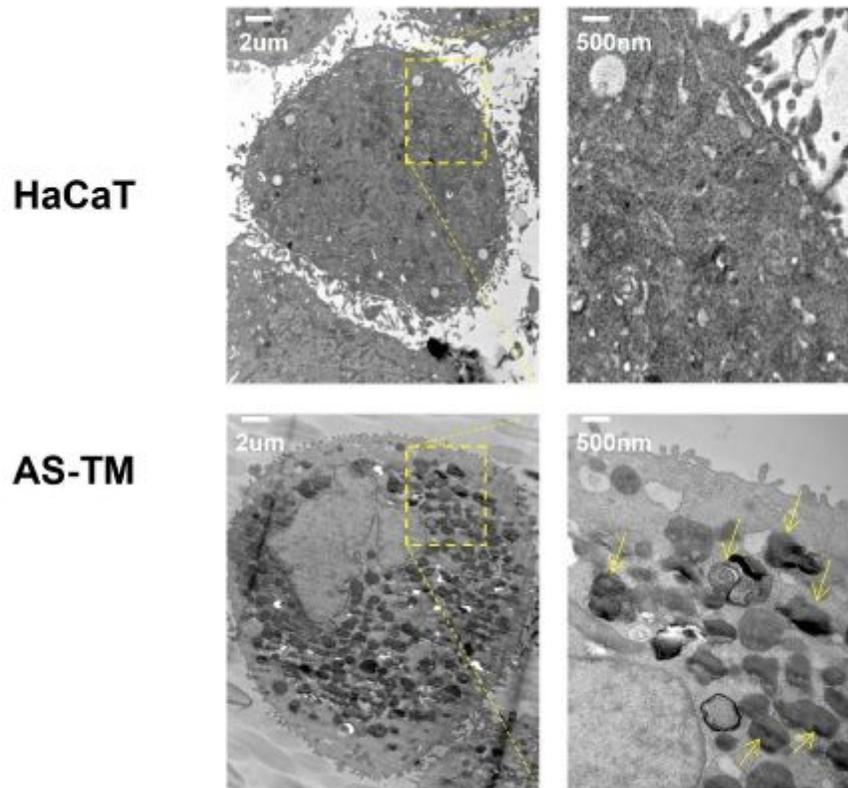


Figure 4

Arsenic treatment causes cell cycle dysregulation through upregulation of p16,Rb,CDK4 and Cyclin D1 (A) After arsenic treatment of HaCaT cells at different times, p16,Rb,CDK4 and Cyclin D1 mRNA expression levels were determined using Real-time PCR. n = 3. * p < 0.05, ** p < 0.01,*** p < 0.001,vs. control cells (0 weeks). (B) Western Blot was applied to detect the Cyclin D1 protein expression levels after 28 weeks of arsenic treatment.

A



B

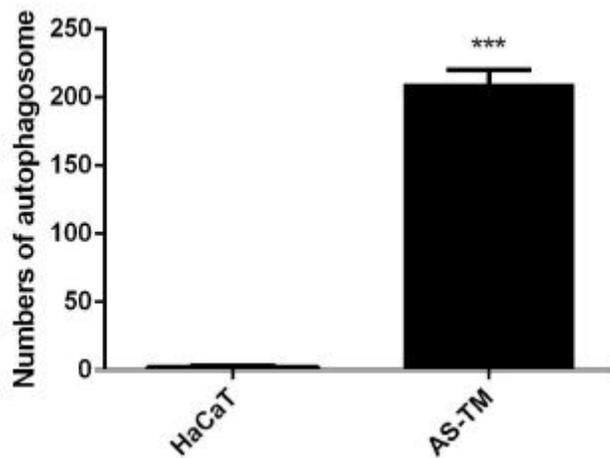


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Arsenic activated autophagy in HaCaT cells (A) TEM detected numerous double-membraned autophagosome-like vesicles in the AS-TM. (B) Autophagosomes were counted from image by TEM (n=3), ***p<0.001.vs. HaCaT.

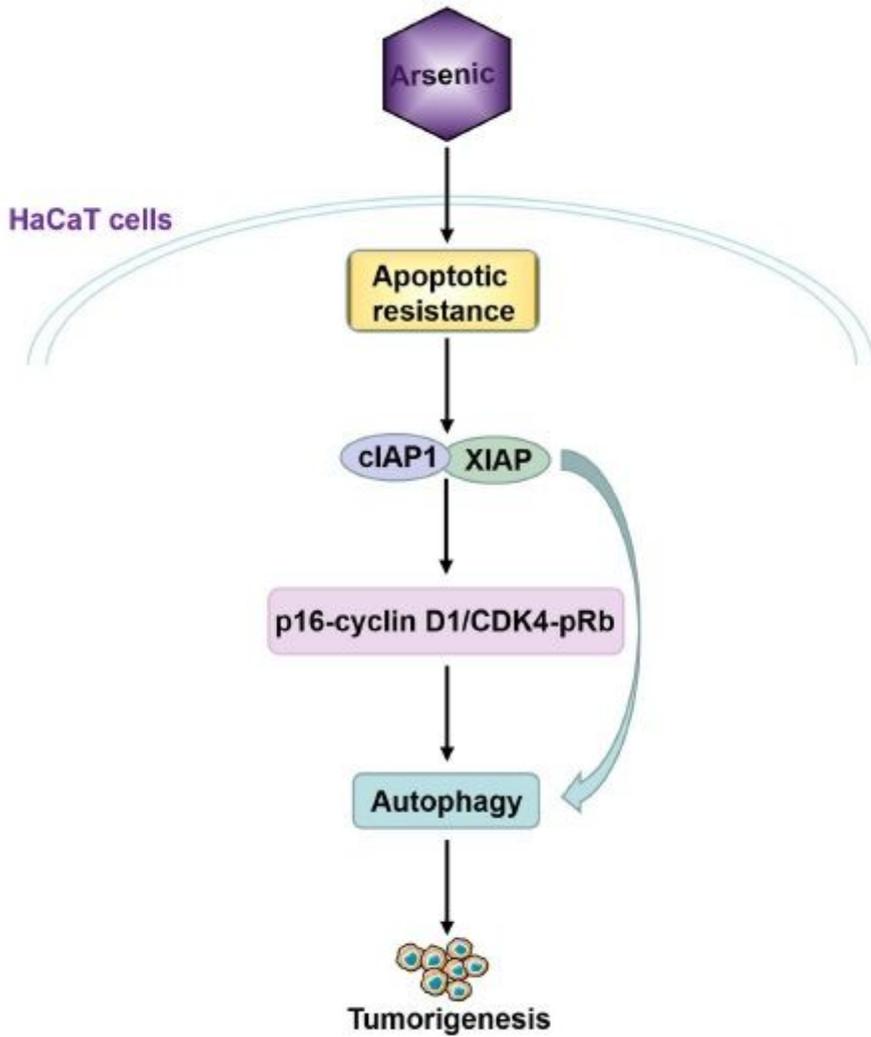


Figure 6

The schematic diagram elucidates the mechanism whereby apoptotic resistance, cell cycle dysregulation and autophagy activation contribute to arsenic carcinogenesis. A low dose of arsenic treatment induce apoptotic resistance by increasing anti-apoptotic proteins XIAP and cIAP1 expression, XIAP and cIAP1 activate autophagy by regulating the p16-cyclin D1/CDK4-pRb pathway. At the same time, XIAP and cIAP1 can also directly activate autophagy, both of them ultimately leading to tumorigenesis.