Dysregulation of autophagy-associated microRNAs in condyloma acuminatum

Shi Wu
department of dermatology, Jinan University
Dan Lu
department of neurology, Jinan University
Xinkai Zheng
department of dermatology, Jinan University
Jin Xu
department of dermatology, Jinan University
Zhen Li
department of dermatology,Jinan University
Liehua Deng (liehuadeng@126.com)
department of Dermatology, Jinan University https://orcid.org/0000-0001-9896-9685
Yunfeng Hu (huyunfeng@jnu.edu.cn)
department of dermatology, Jinan University

Research article

Keywords: Condyloma acuminate, Autophagy, microRNA, cell proliferation, mRNA

Posted Date: March 4th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-15974/v1

Version of Record: A version of this preprint was published at Infection, Genetics and Evolution on September 1st, 2021. See the published version at https://doi.org/10.1016/j.meegid.2021.104878.

Abstract

Objectives This study was designed to investigate the miRNAs that regulate the cell proliferation of condyloma acuminatum (CA) lesions and their targets.

Methods The expression of Ki-67 in 26 CA patients compared with 10 healthy controls was assessed by immunohistochemistry. And the different miRNAs in 4 CA patients and 4 control cases were analyzed by bioinformatics. PCR was used to validate the expression of screened miRNA and its corresponding target genes.

Results The expression of Ki-67 was abnormally increased in CA compared with healthy controls (P <0.05). The comparison of the control group with the CA group revealed 81 differentially expressed miRNAs, of which 56 were downregulated and 25 were upregulated. Two of the differentially expressed miRNAs, miR-30a-5p and miR-514a-3p, are associated with cell proliferation and their target genes are autophagy-related protein (Atg) 5 and Atg12, and Atg3 and Atg12, respectively. PCR results showed that the expression levels of miR-30a-5p and miR-514a-3p were decreased in CA patients compared with healthy controls (P <0.05), whereas the expression of Atg5, Atg12 and Atg3 was increased (P <0.05). The expression of the autophagy proteins microtubule-associated protein 1 light chain 3 (LC3) and P62/SQSTM1 (P62) was abnormally increased in the local lesion tissue of the 26 patients with CA compared with the 10 healthy controls, as assessed by immunohistochemistry (P <0.05).

Conclusions Our results suggest that autophagy levels may be modulated by has-miRNA30a-5p and has-miRNA514a-3p in CA patients, leading to dysregulated cell proliferation.

1. Introduction

Condyloma acuminatum (CA), which is caused mostly by human papillomavirus (HPV) infection, is a kind of benign papilloma hyperplasia disease located in the genital, perineal and anal regions[1]. Recently, CA has become one of the most common sexually transmitted diseases in China[2]. CA is asymptomatic, easily infects, grows rapidly, recurs[3], and is characterized by abnormal local cellular proliferation. However, currently, the specific pathogenic mechanisms underlying the local changes in the vulvar epithelial tissue after HPV infection remain unclear.

Micro RNAs (miRNAs), which are approximately 22nt in length, are a class of endogenous noncoding single-stranded small RNAs in eukaryotic organisms. MiRNAs are important regulators of cellular proliferation. MiR-99b expression is lower in CA samples than healthy control skin samples, and miR-99b plays a role in keratinocyte proliferation by targeting IGF-1R[4]. MiRNAs can also regulate the differentiation process of related mRNAs and key proteins. A recent study demonstrated that the expression of autophagy-related mRNAs in the local tissues of CA patients is abnormal compared with that in the healthy controls[5]. This finding suggests that autophagy may play a role in the pathogenesis of CA.

Autophagy, or cellular self-digestion, is a cellular pathway involved in human physiology and disease. Autophagy is an evolutionarily conserved process that delivers cytoplasmic components to lysosomes for degradation and subsequent recycling of the degradation products. Accumulating evidence shows that autophagy is also involved in many of the pathophysiological processes of human diseases, including microbial infection[6]. Recent studies have shown that during the process of infection, certain viruses can inhibit and/or induce the occurrence of autophagy in the host to enhance their replication. Furthermore, both DNA viruses and RNA viruses can affect the occurrence of autophagy[7]. Although it has been reported that the local autophagy level in CA changes[5], the specific underlying mechanism is not clear. In particular, it is not clear which microRNAs are involved and how they regulate autophagy in CA.

In the current study, we demonstrate that the reduction in miR-30a-5p and miR-514a-3p levels promotes the proliferation of tissue in CA patient lesions, which are due to their ability to regulate the expression of target genes autophagy-related genes(Atg)3, Atg5, and Atg12. This mechanism may affect the occurrence and development of CA on the skin.

2. Materials And Methods

2.1 Patients and specimen selection and biopsies

26 CA cases were selected from the Dermatopathology Department at the First Hospital Affiliated with Jinan University (Guangzhou, China) from 2017 to 2019. Another 5 cases of female false condyloma in the Dermatology Clinic and 5 healthy adult male foreskin epithelial tissues obtained in the Surgical Clinic were included as the control group. The CA lesions and normal epithelia were confirmed by histopathology. And 4 cases of CA tissue and 4 cases of prepuce tissue used for miRNA screening and PCR. The study was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University. All respondents signed an informed consent form. All patients signed informed consent before any biopsies were taken.

2.2 Immunohistochemical (IHC) staining of microtubule-associated protein 1 light chain 3 (LC3), P62 and Ki-67.

Continuous sections were dewaxed and incubated with 0.3% hydrogen peroxide in methanol for 30 min. At room temperature, the nonspecific binding was blocked by incubating the sections in nonimmune serum (diluted in phosphate-buffered saline (PBS) at 1:75) for 20 min. The sections were incubated with the rabbit-anti-human monoclonal anti-LC3 (1:500), P62 (1:400; Wanlei Biotechnology Inc. China) and Ki-67 (1:400; Cell Signaling Technologies, Inc.) antibodies in a humid box for 12 hours at 4 °C. After the excess reagent was removed, the sections were incubated for 30 min with a biotinylated secondary antibody (mouse anti-rabbit) diluted 1:200. Finally, the sections were observed and photographed with an inverted fluorescence microscope (Nikon, Japan).

2.3 Assessment of LC3, P62 and Ki-67 expression

Fig. 2E indicate the positive immunohistochemical expression of LC3 and P62 in the cytoplasm. IHC staining for LC3 and P62 was assessed according to the intensity and proportion. The intensity score was evaluated as follows: 0 (negative staining), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). The area of staining was determined as 0 (no staining of cells in any microscopic fields), 1 (< 30% of the tissue was stained positively) and 2 (> 30% of the tissue was stained positively). The total score was the intensity score multiplied by the proportion score. The total scores were as follows: 0 to 1 (-), 2 to 4 (+) and 5 to 6 (++). The criterias used in this study have been widely accepted previously[8]. The proportion of Ki-67 positive cells was counted using a high-power microscope, 5 high-power microscope fields were analyzed for each sample, and the mean value was taken.

2.4 MiRNA microarray analysis

The miRNA microarray was performed by Shanghai Biotechnology Corporation. In this study, 4 patients were randomly selected from each group. Total RNA was extracted from the vulvar epithelial tissue using mirVanaTM PARISTM (Ambion, Austin, TX, US), and the RIN number was checked on an Agilent Bioanalyzer 2100 to inspect the RNA integrity. The miRNA was labeled by a miRNA Complete Labeling and Hyb kit (Agilent Technologies, Santa Clara, CA, US). Each slide was hybridized with 100 ng of Cy3-labeled RNA using a miRNA Complete Labeling and Hyb kit in a hybridization oven (Agilent Technologies, Santa Clara, CA, US) at 55°C and 20 rpm for 20 hours. Following hybridization, the slides were washed in staining dishes with a Gene Expression Wash Buffer kit. The slides were scanned by an Agilent Microarray Scanner and Feature Extraction software 10.7 at the default settings.

2.5 Quantitative reverse transcriptase PCR assay to detect miRNAs and mRNAs expression

After the differentially expressed miRNAs were identified, the miRNAs associated with cell proliferation (miR-30a-5p, miR-514a-3p) were selected, and the mRNAs regulated by these two miRNAs were found. Finally, the expression of the miRNAs and mRNAs were verified by quantitative reverse transcriptase PCR (qRT-PCR). TRIzol reagent (Life Technologies, US) was used to extract the total RNA according to the manufacturer's instructions. The miRNA reverse transcription procedure was carried out according to the All-in-One^{TM} miRNA qRT-PCR Detection kit (GeneCopoeia, US) instructions. All primers for miRNAs were provided by the Shanghai Biological Engineering Company (Shanghai, China)and and U6 was used as internal control. The primers for miRNAs were: miR-30a-5p: 5'-UGUAAACAUCCUCGACUGGAAG-3' and miR-514a-3p: 5'-AUUGACACUUCUGUGAGUAGA-3'. A Roche Transcriptor First-Strand cDNA Short Synthesis kit (Roche, Switzerland) was used to carry out the reverse transcription reaction according to the manufacturer’s instructions was used to reverse transcription of mRNA, and Roche LightCycler® 480 SYBR Green I Master Mix (Roche, Switzerland) was used for qRT-PCR. All primers for mRNAs were also provided by the Shanghai Biological Engineering Company (Shanghai, China). The forward primer sequences of Atg3, Atg5, and Atg12 were 5'-TGGAAGTGGCTGAGTACCTG-3', 5'-GCCATCAATCGGAAACTCAT-3' and 5'- CAGTCTGTGTTGCAGCTTCC-3'. Finally, the 2−ΔΔCT method was used to calculate the relative expression of miRNA or mRNA.

2.6 Statistical analysis

The experimental data were analyzed using SPSS 13.0 software, and the results were expressed as the mean ± SEM (n>3). One-way ANOVA and Tukey's method were used to evaluate the difference between the experimental group and the control group. A χ^2 or Fisher's exact test was used to evaluate the differences in the group classification data.

3. Results

3.1 Baseline characteristics of patients

The average age of the CA patients and healthy control was 36.0± 8.8 and 31.2± 10.0, respectively. In the CA group, 80.8% of the participants were male, and in the control group, 50% of the participants were male. CA commonly occurs on the prepuce, labium, scrotum, perineum, coronary sulcus and glans of the penis. But in our study, only the foreskin and inner labia as control group epithelial tissue due to the patients' intention. At baseline, there was no significant difference in age, sex or sampling location between the CA and control groups (all P> 0.05, as shown in Table 1).

3.2 HPV might induce the abnormal proliferation of skin tissue in the genital area

According to normal biopsy procedures, tissue samples were cut, a pathological examination was performed, and then the proliferation marker Ki-67[9] in the tissues was labeled by immunohistochemistry (Fig. 1B). CA is clinically characterized by scattered verrucous projections of the vulva, usually without pigmentation. The surface is usually covered with finger-like projections[10]. The pathological features of CA include hyperkeratosis, parakeratosis, papillated epidermal hyperplasia, hypertrophy of the spinous layer, vacuolar cell presence, keratinaceous granule concentration in the granular layer and the spinous layer, vascular expansion of the superficial dermis and infiltration of lymphocyte-dominant inflammatory cells into the blood vessels[10] (Fig. 1A). The rate of Ki-67-positive staining in the CA group and control group was 8.97±0.74% and 2.72±0.67%, respectively. The difference between the two groups was statistically significant (P<0.05; Fig. 1B).

3.3 Microarray identification of differentially expressed miRNAs in epithelial samples from patients with CA

Cell proliferation is regulated by miRNAs, suggesting that miRNAs may play an important role in the pathogenesis of CA. However, there are few reports on the changes in miRNA expression in CA tissues. In this study, gene chip technology was used to detect miRNA expression in the CA group and control group epithelial tissue. Compared with that in the control group, the expression of 81 miRNAs was changed in the CA group (Fig. 1C). Among the 81 miRNAs, 25 had increased expression, and 56 had decreased expression. According to the information in the microRNA.org database (http://www.microrna.org/), we selected 2 of the 81 miRNAs. The expression of these 2 miRNAs, miR-30a-5p and miR-514a-3p, was reduced in the CA group compared with the control group, respectively (Fig. 1C).

3.4 MiRNA expression by qRT-PCR detection

We further confirmed whether the changes in the expression of the two miRNAs in CA tissue were consistent with those observed in the chip results. qRT-PCR was used to analyze the expression of miR-30a-5p and miR-514a-3p. The results showed that the expression of miR-30a-5p and miR-514a-3p was reduced in CA epithelial tissue ($P<0.05$; Fig. 2A). Moreover, the expression of miR-30a-5p and miR-514a-3p was negatively correlated with the expression of Ki-67 (Fig. 2C, D).

3.5 The mRNA expression of target genes and the related proteins LC3 and P62 expression

The target genes of miR-30a-5p and miR-514a-3p are Atg5 and Atg12, Atg3 and Atg12. Therefore, qRT-PCR was used to analyze the expression of mRNAs; the results showed that the expression of Atg3, Atg5, and Atg12 was increased in the CA epithelial tissue ($P<0.05$; Fig. 2B). Atg3, Atg5, and Atg12 genes are associated with autophagy, suggesting that the autophagy level of the local skin tissue in CA patients may be changed. Therefore, we used LC3 and P62 to evaluate the changes in autophagy levels in the local skin tissue of patients with CA. The immunohistochemical results showed that the expression of LC3 in the CA group was - (26.92%), + (69.23%) and ++ (3.85%), while that in the control group was - (70%), + (30%) and ++ (0%). The P62 immunohistochemistry showed that the -, +, and ++ of the CA group was 11.54%, 61.54% and 26.92%, respectively, while that of the control group was 40%, 60% and 0%, respectively. The expression of LC3 and P62 was increased in CA epithelial tissue ($P<0.05$; Fig. 2E, F).

4. Discussion

CA is one of the most common venereal diseases, and abnormal cellular proliferation is its most common feature. Ki-67, as a marker of proliferation[11], expression was significantly increased in the local skin lesions tissue of CA patients (Fig. 1B). The level of Ki-67 protein in local keratinocytes from patients with psoriasis can be reduced by the overexpression of miR-125b-5p or miR-181b-5p[12]. We found that the expression of Ki-67 increased as the expression of miR-30a-5p and miR-514a-3p decreased in the local tissue lesions of CA patients (Fig. 2C, D). These two miRNAs were screened by gene chip (Fig. 1C), and the PCR verification results were consistent with the gene chip results (Fig. 2A). In non-small-cell lung cancer, miR-30a-5p clearly inhibits proliferation, most likely via the EGF signaling pathway[13]. MiR-514a-3p has been shown to inhibit cell proliferation in clear cell renal cell carcinoma by targeting EGFR[14]. This finding suggests that miR-30a-5p and miR-514a-3p may inhibit the proliferation of keratinocytes in the local skin lesions of CA patients.

The target genes of miR-30a-5p and miR-514a-3p are Atg5 and Atg12, and Atg3 and Atg12, respectively. These target genes were identified using http://www.microrna.org/. The expression of Atg3, Atg5 and Atg12 was increased in the local lesion tissue (Fig. 2B). Atg3, Atg5 and Atg12 all play important roles in autophagy. This suggests that autophagy may play an important role in the abnormal proliferation observed in CA. Currently, autophagy has been reported to play a dual role in viral infection: on the one hand, autophagy can degrade and clear intracellular pathogens to protect normal cells from infection; on the other hand, autophagy is involved in the process of virus infection, facilitating viral escape from host

immunity[15]. Yunfeng Hu et al. confirmed that compared with that in normal cervical epithelial tissue, the expression of Beclin1 and LC3 is significantly decreased in cervical squamous cell carcinoma[16]. Meng Jiang et al. showed that HPV infection downregulates the expression of Atgs in CA[5]. However, compared with that in the control group, LC3 and p62 expression was significantly increased in the local vulvar epithelial tissues of the CA group in this study (Fig. 2E, F). The difference in our results may be related to the difference in the skin of the control group we chose. Meng Jiang et al. chose the chest skin of the healthy individuals as the control, whereas we chose the foreskin of the healthy men and the false condyloma of the healthy woman. However, which stage of autophagy is affected in CA needs further study.

The process of mammalian autophagy is divided into six main steps[17]: initiation, nucleation, elongation, closure, maturation and degradation. The elongation of autophagosomes is mediated by two ubiquitin-like conjugation systems (the Atg12 conjugation system and the LC3 conjugation system), and Atg3, Atg5, and Atg12 play an important role in the formation of these two systems[18]. In the Atg12 conjugation system, the C-terminal glycine of the ubiquitin-like protein Atg12 binds to the lysine residue of Atg5 through an isopeptide bond[19]. The Atg12-Atg5-Atg16L complex is preferentially located on the outer membrane of autophagosomes in mammalian cells[20]. In the LC3 conjugation system, the Atg12-Atg5-Atg16L complex is similar to the E3 ligase in the ubiquitin system, Atg7 is similar to the E1 activating enzyme, and Atg3 is similar to the E2 binding enzyme[21]. LC3-I and its homologs bind to phosphatidyl ethanolamine on the cell membrane, promoting the transformation of LC3-I to LC3-II (Fig. 4). In this study, the change in miRNA expression may increase the level of autophagy by regulating the autophagy conjugation system, but further studies are needed.

The genes differentially expressed between the CA and control groups were analyzed by KEGG pathway enrichment analysis, and the differentially expressed pathways were enriched (Fig. 3). The pathways associated with autophagy were found to be the mTOR, Notch, endocytosis, TGF and P53 signaling pathway. TOR dephosphorylation during poliovirus and coronavirus infection activates autophagy, causing Atg5 and Atg12 recruitment to the isolation membrane[22]. The upregulation of the expression of Atg5 is related to Notch signaling downregulation in hematopoietic differentiation[23]. Recent data suggest that several stages of the endocytosis pathway are essential for effective autophagy in mammalian cells[24]. P53[25] and its target genes Isg20L1[26] can regulate the expression of autophagy genes.

In conclusion, in CA patients, miRNA-30a-5p and miRNA-514a-3p can regulate the cells proliferation in local lesions by targeting Atg3, Atg5, and Atg12. The autophagy-associated mTOR and endocytosis signaling pathways are also affected. Therefore, these processes are regulated during the pathogenesis and development of CA. However, further experiments are needed to verify the function of these miRNAs and the downstream pathway.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University. All respondents signed an informed consent form. All patients signed informed consent before any biopsies were taken.

Consent for publication

Not Applicable.

Availability of data and materials

The data sets analyzed in this study are available to the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (NSFC) (NO. 81801150), National Science Foundation of Guangdong Province (NO. 2018A0303130182) and Postdoctoral Research Foundation of China (NO. 2018M643370).

Authors' Contributions

SW and DL designed the experiments. SW, XZ, YH, ZL and YH performed the experiments. SW and ZL analyzed data and SW wrote the manuscript. DL and LD revised the manuscript.

Acknowledgments

We would like to thank the health care workers and volunteers for their participation in this study. We would like to thank American Journal Experts for performing language editing for this manuscript (Reference: 532VG9NB).

Conflicts of Interest

None declared.

References

1. Steben, M. and S.M. Garland, *Genital warts.* Best Pract Res Clin Obstet Gynaecol, 2014. **28**(7): p. 1063-73.
2. Zhao, J., et al., *Immunotherapy of HPV infection-caused genital warts using low dose cyclophosphamide.* Expert Rev Clin Immunol, 2014. **10**(6): p. 791-799.

3. Cardoso, J.C. and E. Calonje, *Cutaneous manifestations of human papillomaviruses: a review.* Acta Dermatovenerologica Alpina Panonica Et Adriatica, 2011. **20**(20): p. 145-154.
4. Li, J., et al., *miR-99b suppresses IGF-1R expression and contributes to inhibition of cell proliferation in human epidermal keratinocytes.* Biomedicine & Pharmacotherapy, 2015. **75**(3): p. 159-164.
5. Jiang, M., et al., *HPV Infection Downregulates the Expression of Autophagy-Related Genes in Condylomata Acuminata.* Dermatology, 2019: p. 1-8.
6. Silva, L.M. and J.U. Jung, *Modulation of the autophagy pathway by human tumor viruses.* Seminars in Cancer Biology, 2013. **23**(5): p. 323-328.
7. Kudchodkar, S.B. and B. Levine, *Viruses and autophagy.* Reviews in Medical Virology, 2010. **19**(6): p. 359-378.
8. Aggelis, et al., *Expression of p53 and PTEN in human primary endometrial carcinomas: Clinicopathological and immunohistochemical analysis and study of their concomitant expression.*
9. Scholzen, T., . and J. Gerdes, . *The Ki-67 protein: from the known and the unknown.* Journal of Cellular Physiology, 2000. **182**(3): p. 311-322.
10. Léonard, B., et al., *A clinical and pathological overview of vulvar condyloma acuminatum, intraepithelial neoplasia, and squamous cell carcinoma.* Biomed Research International, 2015. **2014**(1): p. 480573.
11. Sun, X. and P.D. Kaufman, *Ki-67: more than a proliferation marker.* Chromosoma, 2018. **127**(3): p. 1-12.
12. Zheng, Y., et al., *MiR-125b-5p and miR-181b-5p inhibit keratinocyte proliferation in psoriasis by targeting Akt3.* Eur J Pharmacol, 2019: p. 172659.
13. Zhu, J., et al., *CD73/NT5E is a target of miR-30a-5p and plays an important role in the pathogenesis of non-small cell lung cancer.* Molecular Cancer, 2017. **16**(1): p. 34.
14. Ke, X., et al., *MiR-514a-3p inhibits cell proliferation and epithelial-mesenchymal transition by targeting EGFR in clear cell renal cell carcinoma.* Am J Transl Res, 2017. **9**(12): p. 5332-5346.
15. Abdoli, A., et al., *Autophagy: The multi-purpose bridge in viral infections and host cells.* Reviews in Medical Virology, 2018. **28**(10).
16. Hu, Y.F., et al., *Expressions and clinical significance of autophagy-related markers Beclin1, LC3, and EGFR in human cervical squamous cell carcinoma.* Oncotargets & Therapy, 2015. **8**(default): p. 2243.
17. Kang, R., et al., *The Beclin 1 network regulates autophagy and apoptosis.* Cell Death & Differentiation, 2011. **18**(4): p. 571-80.
18. Radoshevich, L., et al., *ATG12 Conjugation to ATG3 Regulates Mitochondrial Homeostasis and Cell Death.* Cell, 2010. **142**(4): p. 590-600.
19. Moloughney, J.G., et al., *Vaccinia virus leads to ATG12–ATG3 conjugation and deficiency in autophagosome formation.* Autophagy, 2011. **7**(12): p. 1434-1447.
20. Hanada, T., et al., *The Atg12-Atg5 Conjugate Has a Novel E3-like Activity for Protein Lipidation in Autophagy.* Journal of Biological Chemistry, 2007. **282**(52): p. 37298-37302.

21. Levine, B., N. Mizushima, and H.W. Virgin, *Autophagy in immunity and inflammation.* Nature, 2011. **469**(7330): p. 323-35.
22. Wileman, T., *Aggresomes and autophagy generate sites for virus replication.* Science, 2006. **312**(5775): p. 875-878.
23. Cao, Y., et al., *Autophagy Sustains Hematopoiesis Through Targeting Notch.* Stem Cells & Development, 2015. **24**(22): p. 2660.
24. Tooze, S.A., A. Abada, and Z. Elazar, *Endocytosis and Autophagy: Exploitation or Cooperation?* Cold Spring Harbor Perspectives in Biology, 2014. **6**(5): p. a018358.
25. White, E., *Autophagy and p53.* Cold Spring Harbor Perspectives in Medicine, 2016. **6**(4): p. a026120.
26. Eby, K.G., et al., *ISG20L1 is a p53 family target gene that modulates genotoxic stress-induced autophagy.* Molecular Cancer, 2010. **9**(1): p. 95.

Tables

Table 1 Clinical Characteristics of Study Participants

Characteristic	CA (n =26)	Control (n =10)	*P*Value
Age, mean (SE) [range]	36.0 (8.8) [20-55]	31.2 (10.0) [20-50]	0.17
Sex			
Male	21 (80.8%)	5 (50.0%)	0.15
Female	5 (19.2%)	5 (50.0%)	
Location			
Prepuce	9 (34.6%)	5 (50.0%)	0.06
Labium	3 (11.5%)	5 (50.0%)	
Scrotum	2 (7.7%)	0	
Crissum:	4 (15.4%)	0	
Coronary sulcus	4 (15.4%)	0	
Glans penis	4 (15.4%)	0	
HPV infection	Positive	Negative	

Figures

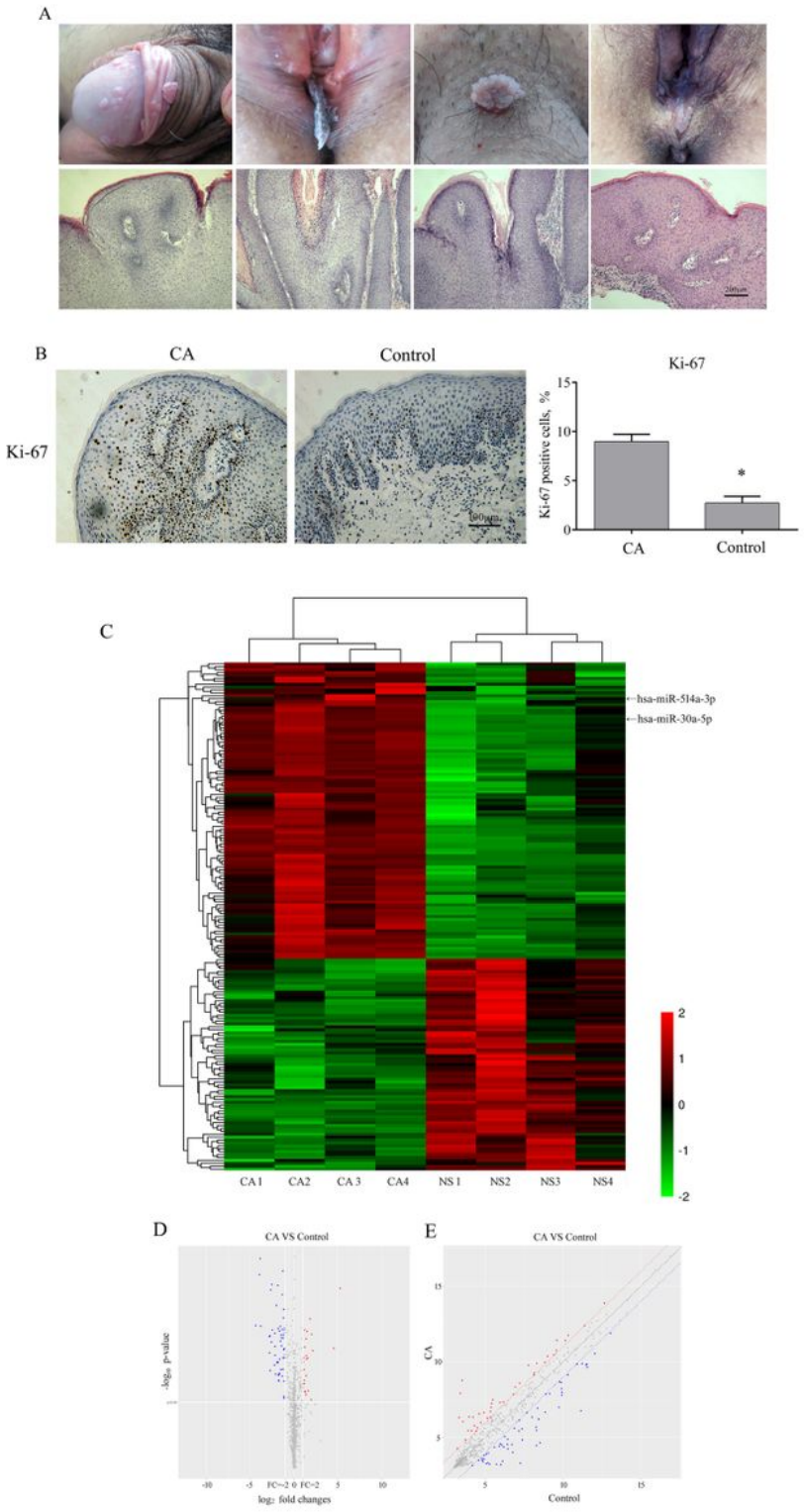

Figure 1

Clinical manifestations, HE staining and Ki-67 expression in local skin lesions and a heat map, volcano plot and scatter plot of miRNAs in CA patients and healthy controls. (A) Clinical manifestations and HE staining of CA tissue. (B) The expression of Ki-67 in CA lesions was higher than that in the control (n>3, P<0.05). (C) The heat map representation of miRNAs differentially expressed in CA patients and healthy controls. The vulvar epithelium of 4 patients with CA and 4 healthy controls was analyzed by miRNA

microarray. A heat map showed that the expression of 25 miRNAs was upregulated >2-fold with $P<0.05$, and the expression of 56 miRNAs was downregulated <0.5-fold with $P<0.05$. Each row represents a miRNA, and each column represents a sample. Each grid color code shows the relative miRNA expression. Two miRNAs further evaluated in this study (has-miR-30a-5p and has-miR-514a-3p) are indicated in the figure. (D) The differential expression of miRNAs in CA patient and healthy control samples is shown by volcano plot. Red area: $P<0.05$ and fold change ≥ 2 differentially expressed genes, and green area: $P<0.05$ and fold change ≤ 0.5 differentially expressed genes. (E) Scatter plot of miRNA expression levels in the CA and control groups. Red plots: upregulated differentially expressed miRNAs, and blue plots: downregulated differentially expressed miRNAs.

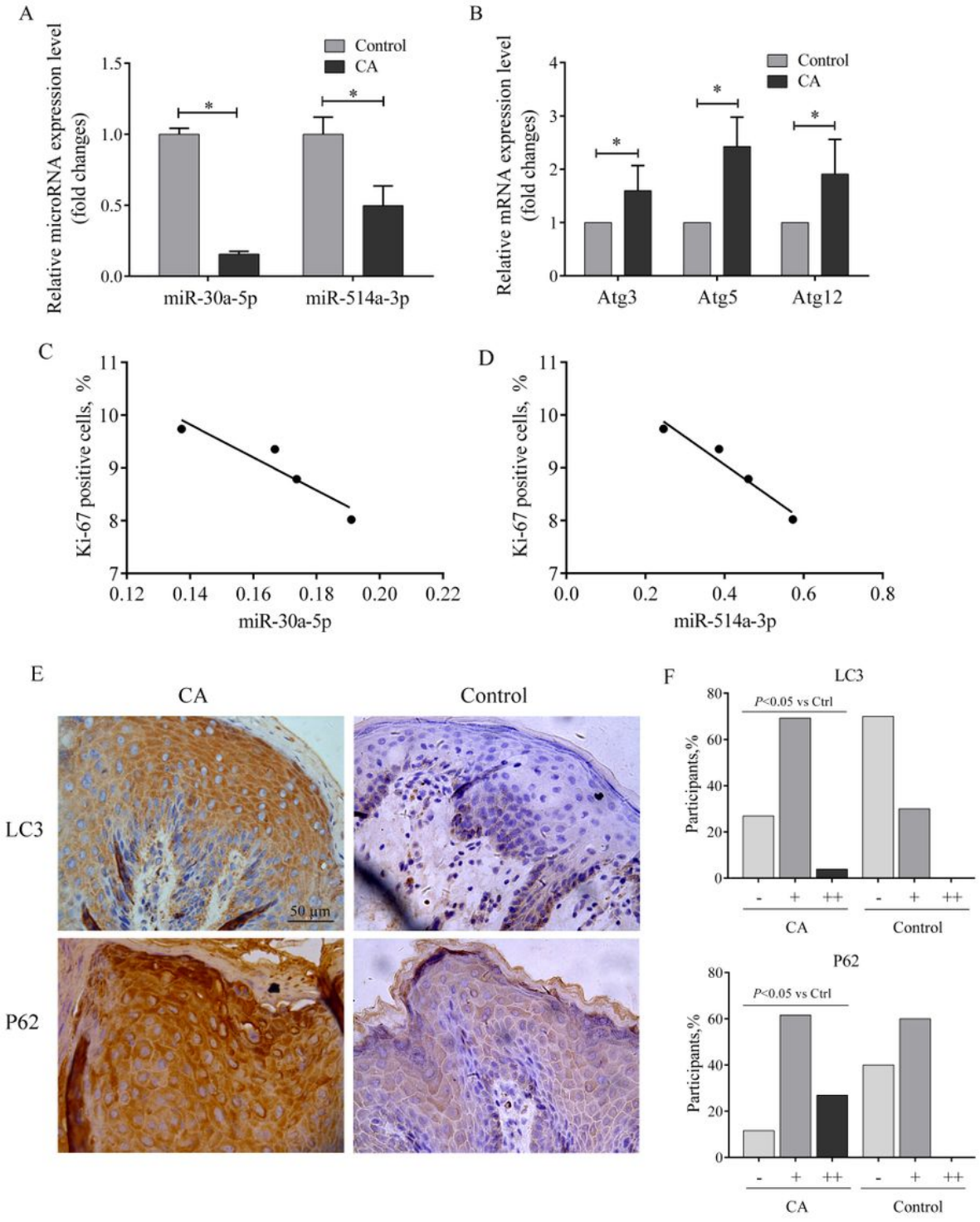

Figure 2

Expression of autophagy-related miRNAs and mRNAs and immunohistochemical staining of LC3 and p62 expression in the CA and control groups, and the relationship between miRNAs and proliferation. (A) The expression of miR-30a-5p and miR-514a-3p was decreased in CA patient epithelial tissue (n>3, P<0.05). (B) The expression of Atg3, Atg5, and Atg12 was increased in CA patient epithelial tissue (n>3, P<0.05). (C, D) The expression of Ki-67 is negatively correlated with miR-30a-5p and miR-514a-3p

expression in CA patient lesion epithelial tissue. (E, F) The expression of LC3 and p62 was greater in the CA group than the control group (n>3, P<0.05).

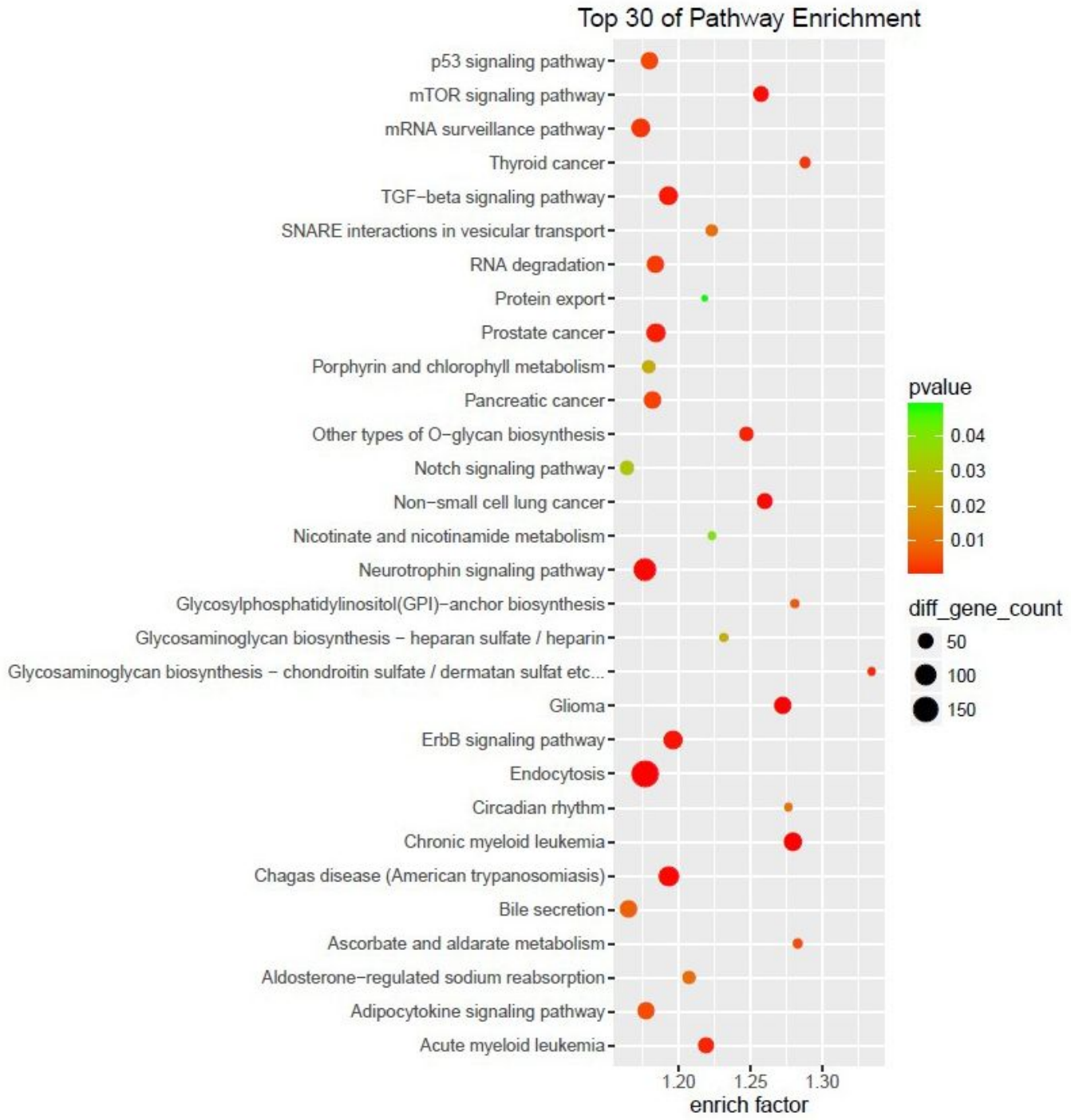

Figure 3

The differentially expressed pathways between the CA and control groups were analyzed by KEGG pathway enrichment analysis.

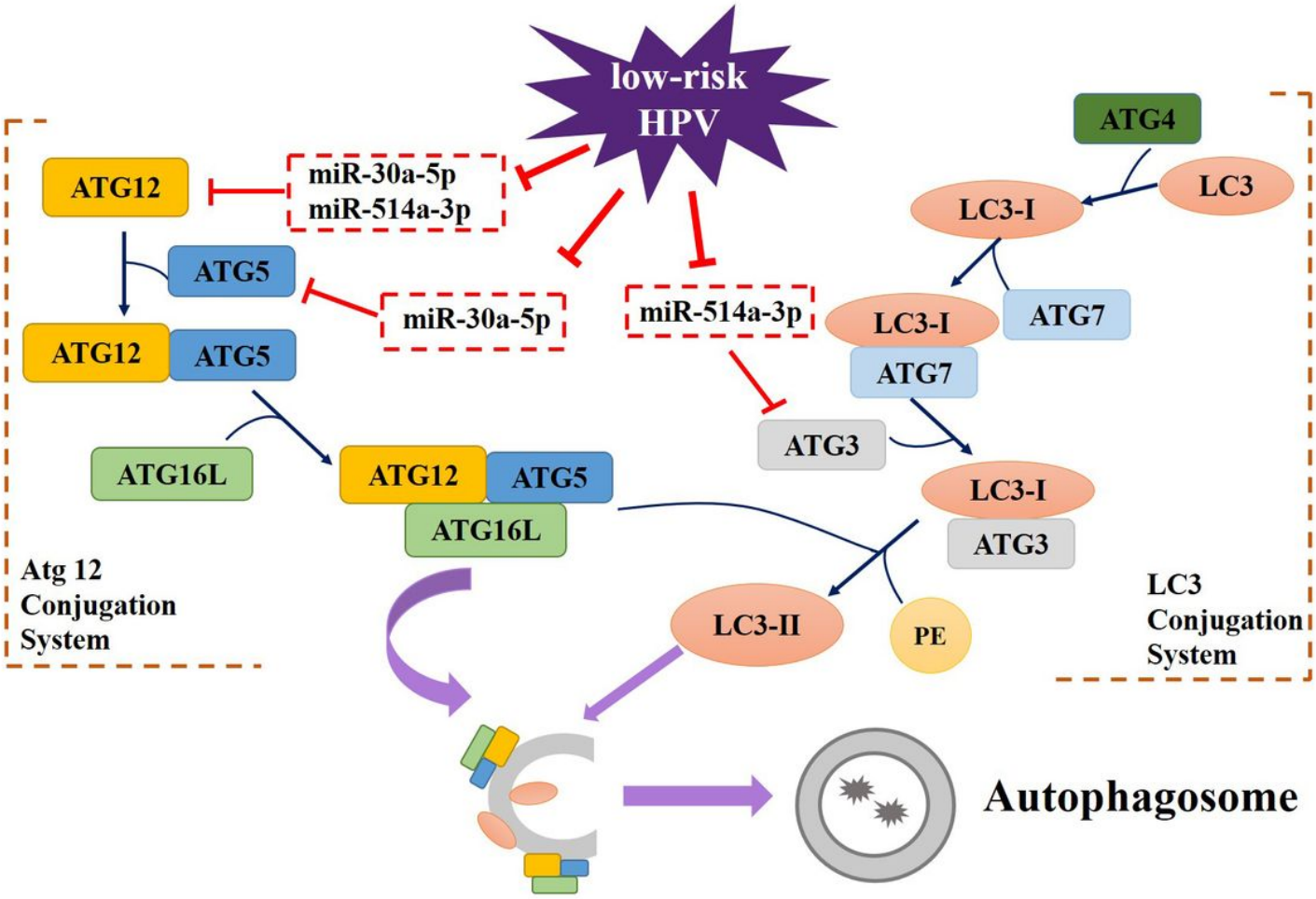

Figure 4

The possible mechanism by which HPV infection affects the formation of local epithelial autophagosomes in CA. CA is induced by HPV. The expression of miR-30a-5p and miR-514a-3p in local epithelial tissues of CA patients is decreased, which leads to the increased expression of Atg3, Atg5, and Atg12 and affects the formation of autophagosomes.