Correlation of miR-125b with Treg/Th17 cell imbalance in Condyloma acuminatum and its regulation on autophagy of keratinocytes

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ABSTRACT

Condyloma acuminatum (CA), a sexually transmitted disease caused by human papillomavirus (HPV) infection, the present research aims to analyze the mechanism of microRNA-125b (miR-125b) in condyloma acuminatum (CA) and its correlation with Treg/Th17 cell imbalance, the objective is to provide new research ideas for the prevention and treatment of CA in the future. The study population comprised 57 CA patients admitted between April 2020 and June 2022 (observation group, OG) and 64 concurrent healthy controls (control group, CG). The peripheral blood miR-125b and Treg/Th17 cells in all participants were detected to identify the correlation of miR-125b with CA severity and Treg/Th17 cells, and the diagnostic value of miR-125b for CA was analyzed. Then keratinocytes (KCs) from skin lesions of CA patients were isolated. Besides, autophagic proteins LC3-II and Beclin-1 in KCs were measured by Western blotting and immunofluorescence staining. miR-125b expression and Th17 cell percentage were lower in OG than in CG, and reduced gradually with the increase of CA severity, while the Treg cell percentage was higher versus CG and increased as CA worsened (P<0.05). miR-125b exhibited a positive association with Th17 cell percentage and an inverse correlation with Treg cell percentage (P<0.05). ROC analysis identified the excellent diagnostic effect of miR-125b on CA (P<0.05). In vitro, increasing miR-125b decreased the ability of KCs to proliferate, enhanced the apoptosis rate, and elevated LC3-II and Beclin-1 expression (P<0.05). In Conclusion, miR-125b, under-expressed in CA patients and explored its relationship with Th17/Treg cell imbalance, the mechanism is related to inhibiting the autophagy of KCs and promoting their abnormal proliferation.

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Introduction

Condyloma acuminatum (CA), a sexually transmitted disease caused by human papillomavirus (HPV) infection, is clinically presented as verrucous lesions, with no complete cure at present (1). CA skin lesions are mainly manifested as skin mucosal vegetations, which are variable in size and shape and often occur in the anus, external genitalia and other parts, with the characteristics of recurrent attacks (2). According to statistics, the risk of developing HPV infection in sexually active people is as high as 80%, and the incidence of CA is about 0.13-0.56% (3). The global prevalence of CA is approximately 160/100,000 to 289/100,000, showing an increasing trend in recent years (4). Hence, it is of great clinical significance for the prevention and treatment of CA. Currently, common clinical treatments for CA include physical therapy (cryotherapy, laser therapy, surgical excision, etc.), chemotherapy (drugs such as 5-fluorouracil and pinyamycin), and immunotherapy (antiviral therapy or immune boosters), but regardless of the treatment, patients are at risk of recurrence (5).

CA has an incubation period of 1-8 months, which makes early identification and prevention quite tricky (6). MicroRNAs (miRNAs) are a hotspot in modern medical research, and their biological characteristics have shown excellent potential in many ways, such as early diagnosis of diseases, prognosis evaluation, and molecular targeted therapy (7, 8). Although the pathogenesis of CA is not yet clear, several studies have shown that microRNAs play an important role in the formation of CA. For example, dysregulation of microRNA autophagy may cause the pathological progression of CA (7). microRNAs can regulate the tension of phosphatases in CA, causing enhanced infectivity of HPV viruses (9). Of them, miR-125b is a gene that has been confirmed to be closely related to HPV and is also known as the key to future diagnosis and treatment of HPV-induced cervical cancer (10). At present, however, research on the correlation of miR-125b with CA is rare. In addition, it is well known that the occurrence of CA is strongly associated with the body's cellular immunity (11). Among them, Th17/Treg cells, as subsets of CD4+ T cells, have important links with CA, AIDS, hepatitis B and other infectious diseases (12, 13). Autophagy is an important process of cellular immunity, referring to the process by which cells engulf their own cytoplasmic proteins or organelles to complete some metabolism or organelle renewal (14). And CA, as a disease associated with immune system disorders, has a close relationship between its pathological development and cellular autophagy (15).

In order to further understand the correlation of miR-125b with CA, this study measured miR-125b expression in CA patients and explored its relationship with Th17/
Treg cell imbalance, so as to provide a more reliable reference for subsequent clinical research.

Materials and Methods

Study participants

The study population comprised 57 CA patients (observation group, OG) who visited our hospital between April 2020 and June 2022 and 64 concurrent healthy controls (control group, CG) with underwent physical examinations. This research has been approved ethically, and all participants provided the informed consent form.

Criteria for patient inclusion and exclusion

OG: patients enrolled all aged 20-35, with complete clinical records and confirmed diagnosis of CA (16) after examination in our hospital. Those with syphilis, AIDS, hepatitis B and other infectious diseases, who lost to follow-up after wart removal treatment, or those with autoimmune disorders were excluded. CG: patients aged 20-35 years, who underwent routine physical examinations in our hospital, with normal examination results and no major medical history were enrolled.

quantitative Real-time Polymerase chain reaction (qRT-PCR)

Peripheral blood samples were taken at admission from both groups. Total RNA from whole blood was isolated with Trizol (ThermoFisher Scientific), and reverse transcription was carried out to obtain cDNA (Reverse Transcription Kit, ThermoFisher Scientific) after verifying its purity using an ultraviolet spectrophotometer. Then amplification was carried out according to the PCR kit (ThermoFisher Scientific) instructions, and the reaction conditions were 95 ℃ for 10 min, 95 ℃ for 15 s and 60s ℃ for 1 min, with 45 cycles in total. MBL BEIJING BIOTECH CO., LTD (Tab 1) was commissioned to design and construct the primer sequences (Table 1). 2-△△CT was employed to calculate miR-125b expression relative to U6.

Flow cytometry (FCM)

Peripheral blood mononuclear cells were isolated and inoculated into cell culture wells, followed by their culture in RPMI culture (Sigma-Aldrich) media and the addition of 5% Tris-buffered saline, and gray value analysis with Image J. Following trypsin inhibitor addition the next day, KCs were pipetted into a single-cell suspension, which was then centrifuged for 5 min to discard the supernatant. KCs were then inoculated into a culture plate and subcultured when they were 70-80% confluent.

Cell transfection

KCs (3×10^4/well) during the logarithmic growth phase were inoculated into the wells of a 6-well plate. After adhering to the wall, they were grouped as follows: miR-125b-min group transfected with miR-125b mimics, miR-125b-inh group transfected with miR-125b inhibitors, and miR-125b-NC group transfected with MiR-125b negative control mimics. The Lipofectamine 2000 kit (ThermoFisher Scientific) instructions were strictly followed during the operation process, all plasmids were designed and constructed by Suzhou Bexin Biotechnology Co.

MTT assay

The transfected cells were prepared into a suspension and inoculated into a 96-well plate (200 μL/well). MTT (Sigma-Aldrich) solution with a volume of 10 μL was added for a 4-hour culture after every 24 hours of incubation. DMSO (20 μL/well) was added to stop the reaction, after which a microplate reader was utilized to measure the absorbance at 570 nm.

Apoptosis rate detected by FCM

The transfected cells were trypsin-digested (Abcam) and rinsed with PBS twice. The apoptosis rate was measured by Annexin-V/PI apoptosis detection kit (Beijing Beyotime Biotechnology), and the results were analyzed by FCM.

Western Blotting detection

BCA (Abcam) was utilized for protein quantification after RIPA lysis (Abcam) of the cells. The proteins were then transferred to a PVDF (Sigma-Aldrich) membrane via SDS-PAGE (Sigma-Aldrich), sealed with 5% skim milk for 2h, and added with LC3-II, Beclin-1 and GAPDH primary antibodies (1:1,000, Abcam). After incubation at 4℃ overnight, an HRP labeled secondary antibody (1:5,000, Abcam) was added to incubate at an ambient temperature for 1h, followed by development using ECL, and gray value analysis with Image J.

Immunofluorescence staining assay

After 15 min of immobilization in 4% formaldehyde, cells were grown for 1h with 5% Tris-buffered saline and Tween-20 diluted skim milk. Following overnight incubation (4 ℃) with primary antibodies, LC3-II and Beclin-1 (1:1,000, Abcam), a second antibody (1:200, Abcam) and DAPI (100 ng/mL, Hangzhou Xiaoyu Biotechnology Co.) were added to incubate for 30 min. A confocal laser scanning microscope was used for observation.

Statistical processing

We employed SPSS 23.0 software for statistical analysis. All independent experiments were performed three times.

<table>
<thead>
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<th>Table 1. Primer sequences.</th>
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<tbody>
<tr>
<td>F (3’-5’)</td>
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<td>miR-125b</td>
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<tr>
<td>U6</td>
</tr>
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times. The Chi-square test was utilized to compare patients’ gender, family history and other count data [n (%)]. miR-125b expression, apoptosis rate and other quantitative data (χ±s) were compared by the paired t-test, and variance analysis and Bonferroni test were used for comparison among groups. Diagnostic value and correlation analyses were performed by the receiver operating characteristic (ROC) curve and Pearson correlation coefficients, respectively. Statistical significance was present when P<0.05.

Results

Clinical baseline data
We found no statistical difference in patients’ general data between OG and CG when comparing the age, sex, family history and other clinical baseline data (P>0.05, Table 2), which proved that the two groups were comparable.

Comparison of miR-125b and Th17/Treg cells
According to the detection, miR-125b expression in OG was (1.76±0.39), which was markedly lower versus CG (P<0.05, Fig 1A). Besides, OG showed an obviously lower percentage of Th17 cells and a higher percentage of Treg cells than CG (P<0.05, Fig 1B), indicating obvious Th17/Treg cell imbalance in CA patients. miR-125b was lowly expressed in the peripheral blood of CA patients, while CA patients had a significant Th17/Treg cell imbalance.

Relationship between miR-125b, Th17/Treg cells and CA
In patients with CA, miR-125b expression and Th17 cell percentage were the lowest in high-risk patients, while the Treg cell percentage was the highest (P<0.05); medium-risk patients presented lower miR-125b expression and Th17 cell percentage while higher Treg cell percentage than low-risk patients (P<0.05, Fig 2A-C). That is, the more severe the CA, the lower the miR-125b expression level and the more severe the Th17/Treg cell imbalance.

Correlation of miR-125b with Th17/Treg cells
Pearson correlation coefficients showed that miR-125b was positively linked to Th17 cell count (P<0.05, Fig 3A), but negatively correlated with Treg cell count (P<0.05, Fig 3B). This indicates a potential link between miR-125b and

Table 2. Clinical baseline data.

<table>
<thead>
<tr>
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<th>CG (n=64)</th>
<th>OG (n=57)</th>
<th>t (χ²)</th>
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<td>36.39±6.28</td>
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<td>0.657</td>
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<td>0.105</td>
</tr>
<tr>
<td>Female/male</td>
<td>59/5</td>
<td>47/10</td>
<td>3.188</td>
<td>0.074</td>
</tr>
<tr>
<td>Family history</td>
<td>3/61</td>
<td>8/49</td>
<td>1.612</td>
<td>0.204</td>
</tr>
<tr>
<td>Have/none</td>
<td>20/44</td>
<td>12/45</td>
<td>0.994</td>
<td>0.319</td>
</tr>
<tr>
<td>Smoking</td>
<td>16/48</td>
<td>10/47</td>
<td>3.247</td>
<td>0.072</td>
</tr>
<tr>
<td>Marriage situation</td>
<td>58/6</td>
<td>45/12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Critical degree</td>
<td></td>
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<td></td>
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</table>
the homeostasis of Th17/Treg.

**Diagnostic value of miR-125b for CA**

ROC analysis of miR-125b expression in OG and CG showed that when peripheral blood miR-125b was less than 2.34, its sensitivity and specificity in diagnosing CA were 96.49% and 51.56%, respectively (P<0.05, Fig 4). This demonstrates the excellent diagnostic effect of miR-125b for diagnosing the occurrence of CA.

**Influence of miR-125b on KC activity**

First, the results of miR-125b expression detection in transfected KCs revealed the highest level in the miR-125b-min group and the lowest level in the miR-125b-inh group (P<0.05, Fig 5A), confirming the success of transfection. According to the subsequent MTT assay, the cell growth ability of miR-125b-mim group was notably lower compared with miR-125b-inh and miR-125b-NC groups, while that of miR-125b-inh group was enhanced versus miR-125b-NC group (P<0.05, Fig 5B). Similarly, FCM results identified that miR-125b-mim group had the highest apoptosis rate, while miR-125b-inh group had the lowest (P<0.05, Fig 5C). That is, inhibition of miR-125b expression promotes the growth of KCs, while elevation of miR-125b expression does the opposite.

**Impact of miR-125b on autophagy in KCs**

LC3-II and Beclin-1 protein levels were the highest among the three groups, while those of miR-125b-inh group were higher when compared to miR-125b-NC group (P<0.05, Fig 6A). Similarly, the fluorescence intensities of LC3-II and Beclin-1 were the lowest in miR-125b-mim group and the highest in miR-125b-inh group, as indicated by immunofluorescence staining (Fig 6B). These results indicate that inhibition of miR-125b expression promotes autophagy in KCs, while elevation of miR-125b expression does the opposite.

**Discussion**

CA, a proliferative disease caused by HPV infection, usually presents no special clinical symptoms, and only a small proportion of patients show clinical and histopathological damage (17). On the other hand, CA has the characteristics of a long incubation period and repeated attacks (18), which requires clinical trials to find an early diagnosis and condition evaluation index suitable for CA as soon as possible to provide a more reliable guarantee for patients' health. miR-125b has not only been confirmed to have a close relationship with HPV infection (19, 20), but also has the function of regulating the immune function of cardiomyocytes (21). In a recent study, it has also been proven to be involved in the formation and development of psoriasis (22). Therefore, this study attempts to confirm the role and preliminary mechanism of miR-125b in CA, thus providing a new direction for the follow-up study of CA.

In the present study, we observed reduced peripheral blood miR-125b expression in OG as compared to CG, with the level decreasing gradually as the severity of CA increased, suggesting that miR-125b may be involved in the occurrence and development of CA. This is also consistent with the results of previous research (23), which can support our experimental results. Besides, Th17/Treg cells in OG were found to be obviously out of balance, confirming the obvious immunosuppression in CA patients. As we all know, Th17 and Treg cells are derived from common cell differentiation precursors. Under normal circumstances,
they antagonize each other to maintain the stability of the body's immunologic balance. But when the body is infected, inflammatory cytokines can promote Th17 cell differentiation, resulting in Th17/Treg cell imbalance (24). The elevated level of Treg cells is an important cause of immune suppression, which can inhibit the Th1/Th2 differentiation and mediate the inflammatory response and immune response of T cells (25). Therefore, as the level of Th17 cells decreases, the body's response to inflammation and chemokines is weakened (manifested as increased Treg), thus prolonging the duration of HPV infection and eventually leading to CA (26). The obvious correlation between miR-125b and Th17/Treg cells identified in our research not only reaffirms the close connection between miR-125b and CA but also supports the regulation of miR-125b on human immune function. Finally, through ROC analysis, we identified the excellent diagnostic effect of miR-125b on the occurrence of CA, which has great potential significance for CA that currently has no reliable blood markers. We believe that in the future, miR-125b examination may effectively improve the early detection rate of CA, assist clinical early and timely targeted treatment of patients, and reduce the potential threat of CA.

To further understand and confirm the correlation of miR-125b with CA, we still need to fully understand the exact mechanism of action of miR-125b. At present, the occurrence of CA has been clinically found to be caused by abnormal KC proliferation after HPV infection (27), while miR-125b has been previously confirmed to be associated with abnormal biological behavior changes of gastric cancer, liver cancer and other cells (28, 29). Therefore, KCs from the skin lesions of patients in OG were isolated and transfected with miR-125b abnormal expression sequences. The results showed that the proliferation ability of KCs was decreased and the apoptosis rate was increased after the expression of miR-125b was increased, while the reverse was true when miR-125b expression was decreased. It is suggested that miR-125b with low expression in CA can promote the abnormal growth of KCs, which is also in line with the results of the above clinical trials and previous studies and can preliminarily confirm the preliminary mechanism of miR-125b involvement in CA (30). In addition, cell autophagy is one of the most important links in the life cycle of KCs. Studies have shown that KCs in the lesions of CA patients usually lose their normal autophagy ability, resulting in a large number of cell accumulation and aggregation, thus forming CA (31). miR-125b has also been shown to influence the autophagy of nasal epithelial cells (32). Therefore, we further examined the autophagy of cells transfected with miR-125b. It also showed reduced LC3-II and Beclin-1 expression in KCs after increasing miR-125b, suggesting weakened autophagy at this time; while decreasing miR-125b promoted KC autophagy. It can be seen that miR-125b can also affect the autophagy of KCs, which lays the foundation for the future molecular targeted therapy of CA by miR-125b.

However, the specific mechanism of miR-125b and CA remains to be further confirmed. Moreover, we need to expand the number of clinical cases and experimental period to further clarify the role played by miR-125b in assessing the progression of CA. In the follow-up, we will carry out a more in-depth and comprehensive study on the relationship between miR-125b and CA, so as to provide a more comprehensive reference for clinical practice.

miR-125b, under-expressed in CA, is closely related to Th17/Treg cell imbalance, which has a potential role in evaluating the occurrence and development of CA. The mechanism is related to inhibiting the autophagy of KCs and promoting their abnormal proliferation. In the future, by detecting miR-125b levels in patients in the clinic, it will be more effective to assess the occurrence and development of CA, and targeted therapeutic drugs by miR-125b may become a new approach to CA treatment.

Conflict of interest
The authors declare to have no conflict of interest.

Consent for publications
The author read and proved the final manuscript for publication.

Availability of data and material
Due to the nature of this research, the participants of this study did not agree to their data being shared publicly, so supporting data is not available.

Authors’ Contribution
Xinxin Ma design, work, statistical analysis and manuscript writing.

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Ethics approval and consent to participate
Not applicable.

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