BUKTI CORRESPONDING

gondo mastutik <gondomastutik@fk.unair.ac.id>

Number assigned to your submission (#APJCP-2105-6861)

1 message

Asian Pacific Journal of Cancer Prevention <journal@waocp.org> Wed, May 5, 2021 at 11:06 AM
To: gondomastutik@fk.unair.ac.id, gondomastutik@gmail.com
Cc: apjcp.copy@gmail.com

Manuscript ID: APJCP-2105-6861

Manuscript Title: **p16INK4A Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection**

Authors: Gondo Mastutik,Alphania Rahniayu,Afria Arista,Dwi Murtiastutik,Nila Kurniasari,Trisniartami Setyaningrum,Anny Setijo Rahaju

Dear **Dr. Gondo Mastutik**

I would like to acknowledge receiving of your manuscript titled "**p16INK4A Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection**". Your manuscript will undergo the review process. You can learn about our review process by visiting APJCP's peer review process page.

Please be sure that the submitted manuscript has not been published previously and will not be submitted elsewhere prior to our decision.

You will be informed of our editorial decision once your manuscript has been reviewed. You can always track your manuscript by login to the APJCP site.

Important Notice: Any future communications (email) about this manuscript should be done through our editorial system. All emails will be answered in 3 to 5 days unless your desired action has been taken place or acted on (you can track the action in our editorial system).

I wish to take this opportunity to thank you for sharing your work with us.

Regards,

Executive Managing Editor of Asian Pacific Journal of Cancer Prevention

gondo mastutik <gondomastutik@fk.unair.ac.id>

APJCP - Your manuscript titled "{manuTitle}" (#APJCP-2105-6861 (R1))

1 message

Asian Pacific Journal of Cancer Prevention <journal@waocp.org> Mon, Jun 28, 2021 at 6:17 PM
To: gondomastutik@fk.unair.ac.id, gondomastutik@gmail.com

Manuscript ID: APJCP-2105-6861

Manuscript Title: p16INK4A Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection

Authors: Gondo Mastutik,Alphania Rahniayu,Afria Arista,Dwi Murtiastutik,Nila Kurniasari,Trisniartami Setyaningrum,Anny Setijo Rahaju

Dear **Dr. Gondo Mastutik**

Your manuscript has been reviewed and reviewers asked for major revision. The comments of the reviewer(s) are included at the bottom of this letter **or** as an attached file(s) to this mail.

Please revise your manuscript accordingly and respond to the reviewer(s) comments in a separate file (a text, doc, or PDF file). In the Response to Reviewer File, provide details about the changes you made to the manuscript (refer to section and paragraph that you made changes in the revised manuscript).

After you make necessary changes please log in to journal's management system and follow the option ***"manuscript needing revision"*** and upload your **revised manuscript and the Response to Reviewer File.**

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Once again, thank you for submitting your manuscript to this journal and we look forward to receiving your revision.

Truly yours,

Editorial Office of Asian Pacific Journal of Cancer Prevention

---------- Important editorial Note----------

- - Reviewer commented file is attached.

- - Your manuscript has been screened for English editing; a recommendation was to be edited thoroughly. After applying the reviewer's comments, please seek help from professional English editors to edit the manuscript. APJCP provide editing services for a flat rate of 200 US dollars per manuscript. The editing service is provided just for APJCP clients.

If you would like to use this service, please in your "response to reviewer file" ask for the service. Otherwise, you need to bring an official certificate from an independent English editing service provider indicating that the manuscript has been edited (the certificate will be available to the public as an auxiliary file in the journal site).

- - Extend your acknowledgment section to include a statement for the following items:

1) Funding statement

2) If it was approved by any scientific Body/ if it is part of an approved student thesis

3) Any conflict of interest

4) How the ethical issue was handled (name the ethical committee that approved the research)

5) Authors contribution

6) Availability of data (if apply to your research)

You must provide a statement for each item.

- - In your revision upload, provide the figures in PowerPoint Slides and tables as Excel file. In both PowerPoint and Excel file, make sure you included the title and footnote of figures and tables.

gondo mastutik <gondomastutik@fk.unair.ac.id>

Acknowledgement of Revision (#APJCP-2105-6861 (R1))

1 message

Asian Pacific Journal of Cancer Prevention <journal@waocp.org> Tue, Jul 6, 2021 at 10:57 AM
To: gondomastutik@fk.unair.ac.id, gondomastutik@gmail.com
Cc: apjcp.copy@gmail.com

Manuscript ID: APJCP-2105-6861 (R1)

Manuscript Title: **p16INK4A Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection**

Authors: Gondo Mastutik,Alphania Rahniayu,Afria Arista,Dwi Murtiastutik,Nila Kurniasari,Trisniartami Setyaningrum,Anny Setijo Rahaju

Date: 2021-05-05

Dear **Dr. Gondo Mastutik**

Thank you for submitting the revised file of your manuscript to the **Asian Pacific Journal of Cancer Prevention**

The Editorial Office will proceed on your manuscript and inform you in the earliest time.

If there is anything else, please do not hesitate to contact us.

Truly yours,

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gondo mastutik <gondomastutik@fk.unair.ac.id>

Manuscript Payment Receipt (#APJCP-2105-6861 (R1))

1 message

Asian Pacific Journal of Cancer Prevention <journal@waocp.org> Thu, Jul 22, 2021 at 5:58 PM
Reply-To: journal@waocp.org
To: gondomastutik@fk.unair.ac.id, gondomastutik@gmail.com

Manuscript ID: APJCP-2105-6861 (R1)

Manuscript Title: p16INK4A Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection

Authors: Gondo Mastutik,Alphania Rahniayu,Afria Arista,Dwi Murtiastutik,Nila Kurniasari,Trisniartami Setyaningrum,Anny Setijo Rahaju

Dear **Dr. Gondo Mastutik**,

Thank you for your payment. Your payment is now confirmed. Your manuscript will be sent back to Executive director for further processing. You will receive a galley during the next 30 days.

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Asian Pacific Journal of Cancer Prevention

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10 messages

Asian Pacific Journal of Cancer Prevention <journal@waocp.org> Wed, Jul 14, 2021 at 6:25 PM
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To: gondomastutik@fk.unair.ac.id, gondomastutik@gmail.com

Manuscript ID: APJCP-2105-6861 (R1)

Authors: Gondo Mastutik,Alphania Rahniayu,Afria Arista,Dwi Murtiastutik,Nila Kurniasari,Trisniartami Setyaningrum,Anny Setijo Rahaju

Dear **Dr. Gondo Mastutik**

The APJCP editorial team is glad to inform you that your manuscript titled **"*p16INK4A Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection*"** has been accepted for publication and will be scheduled for publication as soon as we receive the documentary for processing fee payment.

The processing fee is: **300 US Dollars**

Soon you will receive a PayPal invoice from our partner “EpiSmart Science Vector” by email. You can use your PayPal account or your credit card to pay the invoice.

In case you cannot pay by credit card or PayPal, Please let us know, we try to find you an alternative.

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Payment invoice will be issued upon receiving the payment, however, if you need an invoice before payment, please send us an email and let us know.

You will receive an official acceptance letter when we receive your payment.

Thank you and looking forward to receiving your payment.

Editorial Office,

Asian Pacific Journal of Cancer Prevention

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gondo mastutik <gondomastutik@fk.unair.ac.id> Thu, Jul 15, 2021 at 2:19 PM
To: journal@waocp.org

Dear Editors
Thank you for accepting our manuscript.
If you don't mind, will you allow me to pay by bank transfer using the swift code? For that, I need an invoice before paying it.
How about the cost of editing services (English revision service)?
Can I also get the invoice?
Thank you.

Best regard,
Gondo Mastutik
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APJCP Editor-in-Chief <journal@waocp.org> Thu, Jul 29, 2021 at 12:59 AM
Reply-To: APJCP Editor-in-Chief <journal@waocp.org>
To: gondo mastutik <gondomastutik@fk.unair.ac.id>

Hi Dr. Mastuik,
Did you receive the English editing invoice? The manuscript needs editing. If yes, please make sure you pay, if not you will receive one. Pay as soon as you can and upload the payment slip in the journal website.

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gondo mastutik <gondomastutik@fk.unair.ac.id> Thu, Jul 29, 2021 at 12:05 PM
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Dear Editor,

I have paid by pay pal. The payment receipt I attached here. I got an invoice for 300 US Dollars and I paid 300 US dollars.
I wrote the email before I paid.
Thank you.
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2 attachments

Invoice - 1715 Invoice APJCP Gondo Mastutik.pdf
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Dear Editor,

I have paid by pay pal. The payment receipt I attached here. I got an invoice for 300 US Dollars and I paid 300 US

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I do not know, this payment is for APC or includes english editing. According to the guideline manuscript, Indonesia is a low middle income country, APC is 200 US dollars. But I got an invoice for 300 US Dollars from EpiSmart (1715).
May include the English editing.
Thank you.
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APJCP Editor-in-Chief <journal@waocp.org> Wed, Aug 11, 2021 at 2:25 AM
Reply-To: APJCP Editor-in-Chief <journal@waocp.org>
To: gondo mastutik <gondomastutik@fk.unair.ac.id>

Hi Dr. Mastuik, Indonesia is categorized as Upper middle Income. The 300 US dollars is for APC. Editing cost extra 200 US dollars. Best
[Quoted text hidden]

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gondo mastutik <gondomastutik@fk.unair.ac.id> Wed, Aug 11, 2021 at 5:48 AM
To: APJCP Editor-in-Chief <journal@waocp.org>

Dear Editor,
I have not received the English editing invoice.
I am waiting for it.
Thank you.

Best Regard,
Gondo Mastutik

On Thu, Jul 29, 2021 at 1:00 AM APJCP Editor-in-Chief <journal@waocp.org> wrote:
[Quoted text hidden]

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gondo mastutik <gondomastutik@fk.unair.ac.id> Thu, Aug 12, 2021 at 11:24 AM
To: APJCP Editor-in-Chief <journal@waocp.org>

Dear editor,

I have paid the English editing fee.
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Thank you

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Hi Thank you as soon as we recive from editor, it will be published. I have asked to expedite.
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gondo mastutik <gondomastutik@fk.unair.ac.id> Sat, Aug 21, 2021 at 8:56 AM
To: alphania rahniayu <alphania-r@fk.unair.ac.id>

gondo mastutik <gondomastutik@fk.unair.ac.id>

Paper id APJCP-2105-6861 (R1)

6 messages

gondo mastutik <gondomastutik@fk.unair.ac.id> Tue, Sep 7, 2021 at 3:00 PM
To: APJCP Editor-in-Chief <journal@waocp.org>

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I would like to ask for an update of the article id APJCP-2105-6861 (R1).
Until now, I have not received any information about galley proof.
I hope that the article will be published soon.

Thank you for your attention.

Best wishes,
Gondo Mastutik
=======

Gondo Mastutik
Department of Anatomic Pathology, Faculty of Medicine
Universitas Airlangga, Surabaya, Indonesia.
St. Prof. Dr. Moestopo No 47, Surabaya 60132, Indonesia, Phone: +62-31-5020251 ext 151.
E-mail: gondomastutik@fk.unair.ac.id, gondomastutik@gmail.com

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APJCP Editor-in-Chief <journal@waocp.org> Sat, Sep 11, 2021 at 6:24 AM
Reply-To: APJCP Editor-in-Chief <journal@waocp.org>
To: gondo mastutik <gondomastutik@fk.unair.ac.id>

Hi You will receive very soon.

Editorial Office,
Asian Pacific Journal of Cancer Prevention (APJCP)

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gondo mastutik <gondomastutik@fk.unair.ac.id> Sat, Sep 11, 2021 at 6:36 AM
To: APJCP Editor-in-Chief <journal@waocp.org>

Dear Editor,
Thank you.
I will wait for it.

Best Wishes
Gondo Mastutik
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gondo mastutik <gondomastutik@fk.unair.ac.id> Wed, Oct 6, 2021 at 3:20 AM
To: APJCP Editor-in-Chief <journal@waocp.org>

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Until now, I have not received any information about galley proof.
I hope that the article will be published soon.

Thank you for your attention.

Best wishes,
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APJCP Editor-in-Chief <journal@waocp.org> Wed, Oct 6, 2021 at 4:50 PM
Reply-To: APJCP Editor-in-Chief <journal@waocp.org>
To: gondo mastutik <gondomastutik@fk.unair.ac.id>

Hi Dr. Mustutik, I am very sorry for the delay. It was supposed to be publish in the Sept. Issue however, it came short of reaching to galley stage. But will definatley will be published in Oc

[Quoted text hidden]

[Quoted text hidden]

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gondo mastutik <gondomastutik@fk.unair.ac.id> Wed, Oct 6, 2021 at 5:17 PM
To: APJCP Editor-in-Chief <journal@waocp.org>

Dear Editor,
Thank you for your information. I will wait for it.

Best wishes
Gondo mastutik
[Quoted text hidden]

gondo mastutik <gondomastutik@fk.unair.ac.id>

Request for Submit/Confirm Galley Proof (#APJCP-2105-6861 (R1))

1 message

Asian Pacific Journal of Cancer Prevention <journal@waocp.org> Sun, Oct 17, 2021 at 8:57 PM
Reply-To: journal@waocp.org
To: gondomastutik@fk.unair.ac.id, gondomastutik@gmail.com

Manuscript ID: APJCP-2105-6861 (R1)

Manuscript Title: p16INK4A Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection

Authors: Gondo Mastutik,Alphania Rahniayu,Afria Arista,Dwi Murtiastutik,Nila Kurniasari,Trisniartami Setyaningrum,Anny Setijo Rahaju

Dear **Dr. Gondo Mastutik**,

Your manuscript is in the final stage of publication. The galley proof, official acceptance letter and payment invoice for your manuscript are now ready for download. Please log into your account as the author at http://journal.waocp.org/. In author's page, you have to click on "Galley Proof (1)" and download the galley proof and other files.

The Galley proof is valid only until your paper is published online. It is for proof purposes only and may not be used by third parties.

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Editorial office,

Asian Pacific Journal of Cancer Prevention

gondo mastutik <gondomastutik@fk.unair.ac.id>

Submit/Confirm Galley Proof by Author (#APJCP-2105-6861 (R1))

1 message

Asian Pacific Journal of Cancer Prevention <journal@waocp.org> Tue, Oct 19, 2021 at 10:24 AM
Reply-To: journal@waocp.org
To: gondomastutik@fk.unair.ac.id, gondomastutik@gmail.com

Manuscript ID: APJCP-2105-6861 (R1)

Manuscript Title: p16INK4A Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection

Dear Dr. Gondo Mastutik

Thank you for sending your galley proof. Changes (if asked) will be applied and soon your manuscript will be published in journal's site with "*in* press" status.

Best wishes

APJCP editorial office

gondo mastutik <gondomastutik@fk.unair.ac.id>

Acceptance of Manuscript (#APJCP-2105-6861 (R1))

1 message

Asian Pacific Journal of Cancer Prevention <journal@waocp.org> Wed, Oct 27, 2021 at 7:52 PM
Reply-To: journal@waocp.org
To: gondomastutik@fk.unair.ac.id, gondomastutik@gmail.com

Manuscript ID: APJCP-2105-6861 (R1)

Manuscript Title: **p16INK4A Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection**

Dear **Dr. Gondo Mastutik**

Thank you for your interest in publishing with Asian Pacific Journal of Cancer Prevention. Your manuscript (**APJCP-2105-6861)** is scheduled to be published in **Volume 22, Issue 10, Year 2021**. This Issue will be uploaded into PubMed database around **30th October, 2021**.

Best and thank you for your patience.

Editorial office

Asian pacific Journal of Cancer Prevention

Acceptance letter

Asian Pacific Journal of Cancer Prevention
Official publication of the Asian Pacific Organization for Cancer Prevention

Reference Number: APJCP-2105-6861 Date:10/17/2021

Dear Dr. Gondo Mastutik,

The APJCP editorial board is glad to inform you that the manuscript titled "**p16^{INK4A} Expression in Condyloma Acuminata Lesions Associated with High-Risk Human Papillomavirus Infection**" has been accepted for publication in the Asian Pacific Journal of Cancer Prevention. The Manuscript will be published in our upcoming issue with the following authorship information:

Corresponding author: Gondo Mastutik
First Author: Gondo Mastutik
Listed Co-Authors: *Gondo Mastutik, Alphania Rahniayu, Afria Arista, Dwi Murtiastutik, Nila Kurniasari, Trisniartami Setyaningrum, Anny Setijo Rahaju*

Our production team will soon send you the manuscript's galley proof for your final evaluation.

Thank you for your interest in publishing in APJCP.

"Please be advised that publication of your manuscript may take up to two years. If you think this may not suit your need, just inform us through our editorial system".

SA Mosavi Jarrahi, MSPH, Ph.D.
Editor-in-chief
Asian Pacific Journal of Cancer Prevention

APJCP Scientific rank's among all cancer related journals in Asia for 2015 (Based on Scimago Journal ranking):
Total Citation: Ranks **First** with 9417 citations
H-Index: Ranks **Second** with H-index of 47
SJR (SCImago Journal Rank): Ranks **Seventh** with SJR of 0.813

Reviewer comments

p16^{INK4A} Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection

Gondo Mastutik[*], Alphania Rahniayu[1,2], Afria Arista[3], Dwi Murtiastutik[3,4], Nila Kurniasari[1,2], Trisniartami Setyaningrum[3,4], Anny Setijo Rahaju[1,2]

*Department of Anatomic Pathology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. Email: gondomastutik@fk.unair.ac.id; gondomastutik@gmail.com

Abstract

Objective: The objective was to analyze the correlation between p16^{INK4A} expression and the LR-HPV and HR-HPV infection in condyloma acuminata lesions.

Methods: A cross-sectional study was conducted during January-December 2017 on 33 condyloma acuminata tissues which has been approved by ethical commission. The expression of p16^{INK4A} was detected by immunohistochemistry (IHC) staining. The positive interpretation was carried out by giving a score, namely score 0 (negative), score 1 (sporadic), score 2 (focal) and score 3 (diffuses). The 40 genotypes of HPV were detected by Polymerase Chain Reaction and followed by reverse line blot. The p16^{INK4A} expression data were tested by Mann Whitney, and correlation between variables was analyzed by two-tailed Spearman's rho.

Results: The p16^{INK4A} expression between LR-HPV and HR-HPV groups was significantly different (p = 0,000) and showed the moderate correlation (r = 0,644). LR-HPV in condyloma

Commented [A1]: Major comment: the study includes HPV that is interest to cancer science. However, the English writing is very poor. Needs serious overhauling.

Commented [A2]: No need of ethical clearance in the abstract.

Commented [A3]: Wrong sentence. It should read: A cross-sectional study was conducted during January-December 2017 on 33 condyloma acuminata tissues sample. The study was approved by ethical commission …..

Commented [A4]: Not namely, you do not name you score. English literacy problem

Commented [A5]: No need to bring statistical analysis in the abstract.

Commented [A6]: Define the two group in the method section of the abstract.

acuminata were HPV 6, 11, 42, 61, 54, 81, 87, 89, 90 and HR-HPV 18, 26, 45, 51, 52, 67, 68B, 69, 82. LR-HPV was found in 19/33 patients and HR-HPV was in 14/33 patients. In general, $p16^{INK4A}$ expression showed that 15.2% was diffuse, 24.2% lesion was focal, 39.4% lesion was sporadic, and 21.2% lesion was negative. In LR-HPV group showed that there was no diffuse expression, focal was 15.8%, sporadic was 47.4%, and negative was 36.8%, while in HR-HPV group, it showed that all lesions expressed $p16^{INK4A}$, in diffuse was 35.7%, in focal was 35.7%, and in sporadic was 28.6%.

Conclusion: The most dominant HPV was LR-HPV 11, followed by HPV 6, HPV 18, HPV 51, and HPV 82. The expression of $p16^{INK4A}$ was significantly correlated with HR-HPV infection in which condyloma acuminata lesions infected by HR-HPV have possibility 0.644 times to express $p16^{INK4A}$ compared to infected by LR-HPV.

Keywords: $p16^{INK4A}$, high-risk HPV, low-risk HPV, condyloma

Introduction

Condyloma acuminata is the most frequent sexually transmitted disease that often occurs worldwide (Santegoets et al., 2012). It is also referred to genital warts or anogenital warts. These lesions are usually in single or multiple lesions in the anogenital region accompanied by symptoms of itching, vaginal discharge, and bleeding. The forms are flat or lobulated that looks pearl-like, filiform, plaque eruption, or cauliflower projection (Patel et al., 2013; Léonard et al.,

Commented [A7]: Needs English editing.

Commented [A8]: Grammar problem, it should read: In the LR-HPV group, there was no...

Commented [A9]: Part of result. Conclusion is mostly a qualitative expression of your finding.

Commented [A10]: Wrong word

2014). Its reported prevalence tends to increase. The prevalence of the Italian female population during 2009-2010 was 3.8 cases per 1,000 women per year (Suligoi et al., 2017). In addition, the prevalence in the UK population during 2010-2012 was 3.8% in males and 4.6% in females (Sonnenberg et al., 2019), and in the US population during 2013-2014 was 2.9% (Daugherty et al., 2018). A study systematic review on incident of anogenital warts showed 160-289 per 100,000 persons. New incident of anogenital warts was 103-168 per 100,000 persons among males and 76-191 per 100,000 persons among females (Patel et al., 2013).

Commented [A11]: Wrong sentence in terms of English.

Commented [A12]: English problem

The most common cause of condyloma acuminata is the infection of Human Papillomavirus (HPV). There are two groups of HPV types, including High risk (HR) HPV and Low risk (LR) HPV. HR-HPV are HPV genotypes 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 61, 73, 82 and LR-HPV are HPV genotypes 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 (Braaten et al., 2008; Gutiérrez-Xicoténcatl et al., 2009). LR-HPV causes condyloma acuminata, but study in China showed that infection of HR-HPV was also found in condyloma acuminata lesions. In some cases, condyloma acuminata is caused by combination infection of LR and HR HPV (Lu et al., 2014). Persistent infection of LR or HR-HPV is a risk factor for transformation of epithelial to be benign hyperplasia or to be premalignant lesions. HR-HPV is related to the occurrence of malignancy in women, as cervical cancer (Santegoets et al., 2012). Some literature states that HR-HPV is considered as the main causative agent responsible for cervical cancer (Braaten et al., 2008). The detection of HPV genotype is very important to do to prevent, establish early diagnosis, and perform therapy in cervical cancer. Determination of LR or HR-HPV in anogenital warts can be used as a factor to predict the progression of lesions to be benign or malignant lesions.

The E7 HPV protein plays a role in the cell transformation process. HPV E7 protein binds to important proteins that play a role in the cell cycle such as the pRB and cyclin A/CDK2 complex that inhibits the interaction between Rb and E2F. The E7 protein of HR-HPV will deactivate

pRB resulting in accumulation of $p16^{INK4A}$ protein. The expression of $p16^{INK4A}$ can also be considered as a marker of E7 gene activity (Izadi-Mood al., 2012; Romagosa et al., 2011). $p16^{INK4A}$ plays a role in cell cycle regulation, and is involved in the processes of apoptosis, angiogenesis, cell invasion, and this activity may be associated with overexpression in cancer (Romagosa et al., 2011). The expression of $p16^{INK4A}$ is a marker to determine the prognosis of a malignancy caused by HPV infection (Missaoui et al., 2010). This suggests that $p16^{INK4A}$ is a specific marker for HPV infection and may have a correlation with the type of HR-HPV or LR-HPV. The objective was to analyze the correlation between $p16^{INK4A}$ expression with the LR-HPV and HR-HPV in condyloma acuminata lesions. This study identified the genotype of HPV and performed immunohistochemistry (IHC) staining of $p16^{INK4A}$ expression.

Materials and Methods

The samples collection

A cross-sectional study was conducted at Outpatient Clinic of Department Dermatology and Venereology, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, during period January-December 2017. Ethical study was approved by the Medical Ethic Research from Dr. Soetomo General Academic Hospital Surabaya, number 382/Panke.KKE/V/2016.

The sample inclusion criteria were all patients with condyloma acuminata, both male and female, with a clinical diagnosis of condyloma acuminata and willing to participate in this study by signing the informed consent. The sample exclusion criteria were women who were menstruating and / or pregnant, suffering from an active pelvis and / or acute cervicitis, and men or women with a diagnosis of HIV and AIDS, and someone who was not willing to participate in this study.

The specimens were 33 tissues of condyloma acuminata. Tissue from each patient was divided into 2 parts, one part for tissue processing into paraffin block preparations followed by histopathological diagnosis and immuno-histochemistry (IHC) staining, while another for the examination of the HPV genotype. Histopathological diagnose of condyloma acuminata and analysis of IHC staining was performed by a pathologist.

The expression of $p16^{INK4A}$

The expression of $p16^{INK4A}$ was detected by immunohistochemistry (IHC) staining using Anti-CDKN2A/ $p16^{INK4A}$ Antibody (clone 1E12E10) IHC-plus™ LS-B5261 (LS Bio). The interpretation is positive if cells are stained in the nucleus or combined in the nucleus and cytoplasm. The assessment was carried out as previously reported in Klaes et al. (2001). Score 0 (negative) if cells are stained positive <1% of all cells, score 1 (sporadic) if cells are stained positive <5% of all cells, score 2 (focal) if cells are stained positive <25% of all cells, and the score 3 (diffuses) if the cells are stained positive > 25% of all cells.

The genotyping of HPV

Virus extraction was carried out from condyloma acuminata tissues using the QIAamp DNA Mini Kit (Qiagen) kit, according to the protocol kit. Genotyping of HPV was performed by Polymerase Chain Reaction (PCR), then followed by reverse line blot using the Ampliquality HPV type express v 3.0 kit (Ab Analitica). This kit can detect 40 genotypes of HPV, including HR-HPV which are HPV 16, 18, 26, 31, 33,35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68 (68a, 68b), 69, 73, 82 and LR-HPV which are HPV 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 64, 70, 71, 72, 81, 83, 84, 87, 89, 90.

Statistical analysis

The difference between $p16^{INK4A}$ expression and HR-HPV and LR-HPV genotypes was tested by Mann Whitney test (significant if $p < 0.05$), whereas the correlation between variables was analyzed by two-tailed Spearman's rho (significant if $p < 0.05$).

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Results

Characteristics of patient

This study was performed on 33 tissues obtained from condyloma acuminata patients. Characteristic of patients consisted of sex, age, sexual partner, duration symptom, history of lesion, history of lesion on the partner, type of therapy, efflorescence form, type of lesion, and location of lesion. Patients consisted of 12 (36.4%) males and 21 (63.6%) females, ranging from 18 to 64 years, with the highest frequency was 15-24 years by 17/33 (51.5%) persons. Based on sexual partner, it was dominated by heterosexual (men having sex with women) for 30/33 (90.9%). For bisexual (having sex with the same and different sex), it was 2/33 (6.1%) and homosexual (having sex with the same sex) was 1/33 (3.0%). Based on symptom duration, history of lesion, history of lesion on the partner, and type of therapy, the highest frequency was lesion 1-3 months, the first-time lesion, no lesion on their partner, and received TCA therapy, respectively. Base on the efflorescence form, type of lesion, and location of lesion, the highest frequency was papule form, multiple lesions, and were located in gland penis for male and labia majora for female (Table1).

Commented [A14]: Be care full that you studied patients not tissue. In fact, the unite of your study was patients with wart. You sample tissue from patient. The English is weak and the expression is not fully understood.

HPV genotype in condyloma acuminata lesions

The genotype of HPV included infection of HPV HR and LR, both single and multiple infections. The single or multiple infections of HPV LR was assumed as infection of LR- HPV. The multiple infection of LR-HPV with HR-HPV was assumed as infection of HR-HPV. The genotype of LR-HPV were HPV 6, 11, 42, 61, 54, 81, 87, 89, 90 and HR-HPV were 18, 26, 45, 51, 52, 67, 68B, 69, 82. LR-HPV was found in 19/33 (57.6%) patients and HR-HPV were 14/33 (42.4%) patients (Table 2). The most dominant HPV was HPV 11 that infected 24/56 (42.9%) times, then followed by HPV 6 (16.1%) and HPV 18, HPV 51, and HPV 82 for 5.4%, respectively.

$p16^{INK4A}$ expression in condyloma acuminata lesions

The expression of $p16^{INK4A}$ in condyloma acuminata lesions showed that nucleus or the combination of nucleus and cytoplasm of cell was stained in brown color, that indicating in sporadic, focal, or diffuse (Figure 1). The results showed that 7/33 (21.2%) lesion were negative, 13/33 (39.4%) lesions were sporadic, 8/33 (24.2%) lesions were focal, and 5/33 (15.2%) were diffuse. In LR-HPV group, the results showed that negative, sporadic, and focal were 7/19 (36.8%), 9/19 (47.4%), and 3/19 (15.8%), respectively. In HR-HPV group showed that all lesions expressed $p16^{INK4A}$, in sporadic was 4/14 (28.6%), in focal was 5/14 (35.7%), and in diffuse was 5/14 (35.7%) (Table 3). There was a significant different of $p16^{INK4A}$ expression in condyloma acuminata lesion between LR-HPV and HR-HPV groups (p = 0,000). Correlation between $p16^{INK4A}$ expression with LR-HPV and HR-HPV showed moderate correlation (r = 0,644, p = 0,000).

Discussion

166 The microscopic feature of condyloma acuminata lesions by haematoxylin eosin (HE) staining
167 appear as parakeratosis, hyperkeratosis, hypergranulosis, basal cell hyperplasia, and koilocytic
168 (Léonard et al., 2014). In this study, condyloma acuminata lesions were seen as papules, cauli
169 flowers, and flat-tapping papules. The microscopic feature shown are hyperkeratosis,
170 parakeratosis, papillomatosis, hypergranulosis, and hyperplasia of basal cell, as well as the
171 koilocytes that usually accompany HPV infection.

172 The most common causes of condyloma acuminata is infection by HPV. There are more than
173 40 genotypes of HPV that can infect in the anogenital area that are usually infected by LR-HPV
174 including HPV 6 and HPV 11 in single infection, but most commonly co-infection with LR-
175 HPV or HR-HPV (Léonard et al., 2014; Hasanzadeh et al., 2019). This study found all tissues
176 from patients clinically diagnosed as condyloma acuminata were positive for LR-HPV or HR-
177 HPV, in single infection or multiple infection. Most of patients were infected by LR-HPV,
178 while 42.4% were infected by LR-HPV that were co-infected by HR-HPV. This study is in
179 accordance with cross-sectional study in Kuwait, from 156 patients with genital warts showed
180 that 102/156 (65.4%) patents were infected by LR-HPV and 54/156 (34.6%) patients were
181 infected by HR-HPV, in single infection of 88.4% and multiple infection of 11.6% (Al-Awadhi
182 et al., 2019). Study in Spain showed that LR-HPV were 63/138 (45.6%) and 71/138 (41.4%)
183 anogenital warts patients were infected by HR-HPV (Arroyo et al., 2016). Another study found
184 that from 66 anogenital warts specimens, it showed that LR-HPV infected 42/66 (62.1%) which
185 was dominated by HPV 6 (47%), and HPV-11 (13.6%), as well as HPV 18 and HPV 3
186 (Ozaydin-Yavuz et al., 2019). This study indicates that beside LR-HPV, condyloma acuminata
187 is also co-infected with HR-HPV that can develop malignant cancer. Therefore, identification
188 of HPV genotype can predict the risk of developing the diseases. In addition, determination of
189 genotype of HPV has an impact on treatment management, follow-up the outcome of diseases,
190 and prevention strategies

This study obtained LR-HPV and also HR-HPV, which 57.6% of condyloma acuminata patients were infected by LR-HPV and the rest were infected by multiple infection of LR-HPV and HR-HPV infection. The most common of HPV genotypes were HPV 11 and HPV 6 for LR-HPV, followed by HPV 18, HPV 51, and HPV 82 for HR-HPV. The incidence of condyloma acuminata and the malignant progression can be prevented by vaccination. Recently, there are 3 commercial vaccines against HPV infection that Gardasil prevents to infection of HPV 6, 11, 16, 18; Cervarix prevents to infection of HPV 16, 18, and Gardasil 9 prevent to infection HPV 6, 11, 61, 18, 31, 33, 45, 52, 58 (Gupta et al., 2017). The previous study on benign lesion of anogenital showed HPV genotype in condyloma acuminata patients was dominated by HPV 11 and HPV 6 from 13 female patients (Arista et al., 2019) and from 12 male patients (Murtiastutik et al., 2019). In pre-cancerous lesion and cancerous lesion of uterine cervix, those were dominated by HPV 16 (62.68%), then followed by HPV 18 (20.9%), HPV 45 (5.97%), 52 (5.97%), and 67 (4.48%) (Mastutik et al., 2018). Vaccination programs is expected to reduce the incidence of these diseases, but there were found some genotype of HPV that could not covered by the current vaccine. Therefore, strategies to prevent the incidence of condyloma acuminata or progression of malignancy still need to be developed.

Oncoprotein E6 and E7 of HR-HPV play a role in cancer development. HR-HPV E6 mediates p53 inactivation by binding to the conserved domains of E6AP (E6-linked protein) to form the E6/ E6AP/p53 complex. This complex causes degradation of p53 by ubiquitination mechanism. HR-HPV E7 targets to degraded the retinoblastoma protein (pRB). In normal cells, when cells are prevented from entering the S phase, the pRB binds to the E2F family of transcription factor, so that the cell stops at the checkpoint of G1-S phase, activating cell cycles arrest. pRb which is phosphorylated by cyclin D1/CDK 4/6 complex causes E2F released and enter the nucleus. The cell enters the S phase which then starts the activation of gene transcription (Munger et al., 2013, Pal and Kudu, 2020). Furthermore, phosphorylated pRB is a $p16^{INK4A}$ feedback

mechanism. HPV E7 induces degradation of pRB by a ubiquitin proteosome pathway that causes the loss feedback mechanism of $p16^{INK4A}$ and leads to accumulation of $p16^{INK4A}$ that presents as overexpression of $p16^{INK4A}$ (Lassen et al., 2009; Faraji et al., 2017).

The overexpression of $p16^{INK4A}$ in this study was sporadic (39.4 %), focal (24.2%), diffuse (15.2%), dan negative (21.2%). Another study of $p16^{INK4A}$ expression in 24 condyloma acuminata specimens showed variable in which 11/24 (45.8%) were sporadic, 7/24 (29.2%) were focal, and 6/24 (25%) were negative (Kazlouskaya et al., 2013). The expression of $p16^{INK4A}$ in cervix tended to increase from cervical normal epithelium to invasive cervical cancer (Missaoui et al., 2010; Izadi-Mood et al., 2012) and cervical adenocarcinoma (Mastutik et al., 2021). The expression of $p16^{INK4A}$ in HR-HPV showed all specimens were positive ranging from sporadic to diffuse. In LR-HPV were sporadic and focal, whereas 36.8 % of specimens were not expressed in $p16^{INK4A}$ and none of specimens expressed in diffuse category. Other study found in anal lesion with HR HPV infection showed all specimens expressed $p16^{INK4A}$ in ranging from sporadic to diffuse, whereas those infected with LR-HPV were mostly patchy (Leeman et al., 2019), in oropharyngeal squamous cell carcinomas and tonsillar dysplasia that was positive for HPV 16, was also diffuse, but in benign and pre-malignant lesions were positive for HPV 6 and HPV 11 showed variations from negative to strong positive (Mooren et al., 2014). All cervical lesions with HR-HPV had a significant $p16^{INK4A}$ expression with a strong and diffuse expression, whereas that with LR-HPV showed mild expression (score 1) (Missaoui et al., 2014).

The finding highlight from this study is the expression of $p16^{INK4A}$ significantly correlated with HR-HPV infection in which condyloma acuminata lesions infected by HR-HPV have possibility 0.644 times to express $p16^{INK4A}$ compared to condyloma acuminata lesions infected by LR-HPV. As previous studies showed that $p16^{INK4A}$ expression concordance with HR-HPV infection in oropharyngeal squamous (Liu et al., 2015), in mucosal squamous cell carcinomas

241 of the head and neck (Antonsson et al., 2015), in cervical squamous intraepithelial lesion (Yildiz
242 et al., 2007), and invasive cervical carcinoma and vagina (Missaoui et al., 2010; Missaoui et
243 al., 2014). HR-HPV is integrated into the host cell genome, whereas LR-HPV prefers extra
244 chromosome as episome so that the expression of E6 and E7 oncoproteins are within the
245 regulatory framework of E1 and E2 HPV (Boulet et al., 2007). HR-HPV, such as HPV 18, 33,
246 HPV 51, HPV 58, HPV 59 was found to be integrated, while HPV 30, 35, 39, 44, 45, 53, 56,
247 59, 74 and 82 were not integrated but were in episome form (Nkili-Meyong et al., 2019). In this
248 study found HR-HPV 18, 51 and 82. As reported in Nkili-Meyong et al. (2019) that HPV 18
249 was founded to be integrated in 55% of the positive HPV 18 specimen, and HPV 51 was
250 integrated in 25% of the positive HPV 51 specimen, and HPV 82 was founded in extra
251 chromosome. This integration was associated with partial or total deletion of E1 and E2 gene,
252 leading to overexpression of E6 and E7 due to loss feedback mechanism by E2 protein
253 (Woodman et al., 2007; Nkili-Meyong et al., 2019). In addition, the HR-HPV E7 oncoproteins
254 have the higher affinity to bind pRB than LR-HPV E7 that increase the accumulation of
255 $p16^{INK4A}$. Therefore, all specimens infected by HR-HPV in this study showed positive for the
256 expression of $p16^{INK4A}$ showing in sporadic, focal, and diffuse, while LR-HPV showed in
257 sporadic and focal, and no diffuse expression.

258 In conclusion, this study found significant differences between expression of $p16^{INK4A}$ in
259 condyloma acuminata infected by LR-HPV (HPV 6, 11, 42, 61, 54, 81, 87, 89, 90) and HR-
260 HPV (HPV 18, 26, 45, 51, 52, 67, 68B, 69, 82). This $p16^{INK4A}$ expression is correlated with
261 HR-HPV infection in moderate. IHC is a routine method to perform the diagnostic of
262 histopathological. Therefore, IHC of $p16^{INK4A}$ could be used as biomarker for HR-HPV
263 infection that might be useful to predict the malignancy development of condyloma acuminata
264 lesions.

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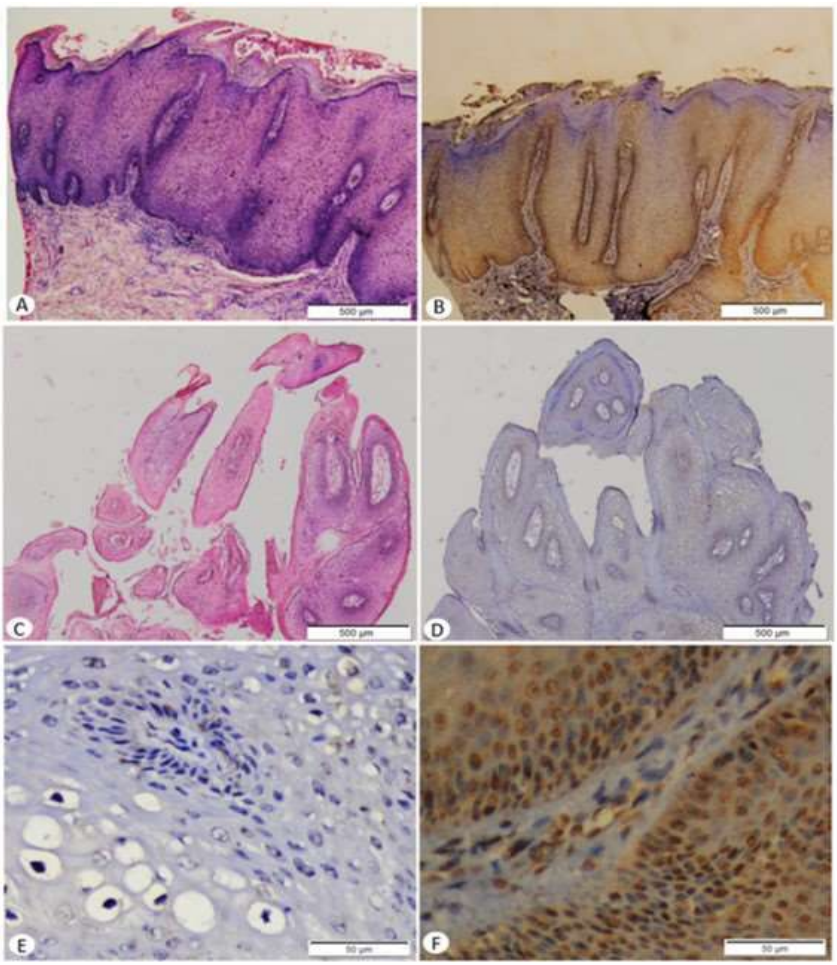

Figure 1. The histopathological feature and $p16^{INK4A}$ expression of condyloma acuminata. A) Condyloma acuminata from HR-HPV infected patients, in HE staining (40 x magnification); B) In the same patients, $p16^{INK4A}$ expression showed in diffuse (score 3) (40 x magnification); C) Condyloma acuminata from LR-HPV infected patients, in HE staining (40 x magnification); D) In the same patients, $p16^{INK4A}$ expression showed negative (score 0) (40 x magnification); E) $p16^{INK4A}$ expression in negative (score 0) (400 x magnification); F) $p16^{INK4A}$ expression in diffuse (score 3) (400 x magnification).

402 Table 1. Characteristics of patients with Condyloma Acuminata

Characteristics of Patients	N (%)	Single or multiple infection LR/LR HPV N (%)	Multiple infection of LR/HR HPV N (%)
Sex:			
- Male	12 (36.4)	7 (21.2)	5 (15.1)
- Female	21 (63.6)	12 (36.4)	9 (27.3)
Age:			
- 15-24 years	17 (51,5)	10 (30.3)	7 (21.2)
- 25-34 years	6 (18,2)	3 (9.1)	3 (9.1)
- 35-44 years	7 (21,2)	4 (12.1)	3 (9.1)
- 45-54 years	2 (6,1)	1 (3.0)	1 (3.0)
- 55-64 years	1 (3,0)	1 (3.0)	0
Sexual Partner			
- Heterosexuals	30 (90,9)	18 (54.5)	12 (36.4)
- Homosexual	1 (3,0)	1 (3.0)	0
- Bisexual	2 (6,1)	0	2 (6.1)
Duration of symptom			
- < 1 month	5 (15,1)	1 (3.0)	4 (12.1)
- 1- 3 months	20 (61,6)	14 (42.4)	6 (18.2)
- 4 - 6 months	6 (18,2)	3 (9.1)	3 (9.1)
- > 6 months	2 (6,1)	1 (3.0)	1 (3.0)
History of lesion			
- First lesion	26 (78,8)	15 (45.4)	11 (33.3)
- Recurrent lesion	7 (21,2)	4 (12.1)	3 (9.1)
Lesion on the partner			
- Have lesion on the partner	4 (12,1)	2 (6.1)	2 (6.1)
- No lesions on the partner	29 (87,9)	17 (51,5)	12 (36.4)
Type of therapy			
- TCA	19 (57,6)	11 (33.3)	8 (24.2)
- Cautery	14 (42,4)	8 (24.2)	6 (18.2)
Efflorescence form			
- Papule	28 (84,8)	16 (48.5)	12 (36.4)
- Cauli flower	4 (12,1)	2 (6.1)	2 (6.1)
- Flat tapped papule	1 (3,0)	1 (3.0)	0
Type of lesion			
- Multiple	31 (93,9)	18 (54.5)	13 (39.4)
- Solitary	2 (6,1)	1 (3.0)	1 (3.0)
Location of lesion			
- Penile	7 (21,2)	4 (12.1)	3 (9.1)
- Anus	4 (12,1)	2 (6.1)	2 (6.1)
- Penile and anus	1 (3.0)	1 (3.0)	0
- Labia (majora and minora)	16 (48,5)	10	6 (18.2)
- Vulva	3 (9,1)	1 (3.0)	2 (6.1)
- Introitus vagina	1 (3.0)	0	1 (3.0)
- Perineum	1 (3.0)	1 (3.0)	0

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404 Table 2. Distribution of HPV in condyloma acuminata lesions

Genotype of HPV	HR or LR HPV genotypes	Frequency
LR HPV		19 (57,6%)
- HPV 6	LR	4
- HPV 11	LR	12
- HPV 6, 11	LR	2
- HPV 6,81,87,89	LR	1
HR HPV		14 (42,4%)
- HPV 6,11,18,51,82	HR	1
- HPV 6,42,51,61	HR	1
- HPV 11, 18	HR	2
- HPV 11,18,45	HR	1
- HPV 11, 26	HR	1
- HPV 11,67	HR	1
- HPV 11, 51, 82	HR	1
- HPV 11, 52, 54	HR	1
- HPV 11, 52,69,90	HR	1
- HPV 11, 68B	HR	1
- HPV 11, 82	HR	2
Total		33

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419 Table 3. The p16 ^{INK4A} expression on condyloma acuminata that infected by LR or HR HPV

p16 ^{INK4A} expression	HPV genotype		Percentage (%)	p-value
	LR-HPV	LR/HR-HPV		
Score 0 (Negative)	7 (36,8%)	0 (0%)	7 (21,2%)	
Score 1 (Sporadic)	9 (47,4%)	4 (28,6%)	13 (39,4%)	0,000
Score 2 (Focal)	3 (15,8%)	5 (35,7%)	8 (24,2%)	
Score 3 (Diffuse)	0 (0%)	5 (35,7%)	5 (15,2%)	
Total	19 (100%)	14 (100%)	33 (100%)	

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Menjawab comments reviewer

p16^{INK4A} Expression in Condyloma Acuminata Lesions correlated with High-Risk Human Papillomavirus Infection

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Abstract

Objective: The objective was to analyze the correlation between p16^{INK4A} expression and the LR/HR-HPV infection in condyloma acuminata lesions.

Methods: A cross-sectional study was conducted during January-December 2017 on 33 condyloma acuminata patients. The expression of p16^{INK4A} was detected by immunohistochemistry (IHC) staining. The positive interpretation was carried out by scoring which score 0 was negative, score 1 was sporadic, score 2 was focal, and score 3 was diffuses. The HPV genotypes were identified by reverse line blot that detected 40 genotypes of HPV, including HR-HPV (HPV 16, 18, 26, 31, 33,35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68a, 68b, 69, 73, 82) and LR-HPV (HPV 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 64, 70, 71, 72, 81, 83, 84, 87, 89, 90).

Results: The expression of p16^{INK4A} was significantly correlated with HR-HPV which patients infected by HR-HPV have possibility 0.644 times to express p16^{INK4A} compared to infected by LR-HPV. LR-HPV in condyloma acuminata were HPV 6, 11, 42, 61, 54, 81, 87, 89, 90 and

HR-HPV were HPV 18, 26, 45, 51, 52, 67, 68B, 69, 82. LR-HPV was found in 19/33 patients and HR-HPV was in 14/33 patients. The expression of $p16^{INK4A}$ in condyloma acuminata lesions showed that 15.2% patients were diffuse, 24.2% patients were focal, 39.4% patients were sporadic, and 21.2% patients were negative. In LR-HPV group, there was no diffuse expression, focal was 15.8%, sporadic was 47.4%, and negative was 36.8%, while in HR-HPV group, all lesions expressed $p16^{INK4A}$, in diffuse was 35.7%, in focal was 35.7%, and in sporadic was 28.6%.

Conclusion: IHC is a routine method in histopathological diagnosis, therefore the detection of p16INK4A expression by IHC can be used as a biomarker for HR-HPV infection.

Keywords: $p16^{INK4A}$, high-risk HPV, low-risk HPV, condyloma, sexually transmitted diseases

Introduction

Condyloma acuminata is the most frequent sexually transmitted disease that often occurs worldwide (Santegoets et al., 2012). It is also referred to genital warts or anogenital warts. These lesions are usually in single or multiple lesions in the anogenital region accompanied by symptoms of itching, vaginal discharge, and bleeding. The type of shape is flat or lobulated that looks pearl-like, filiform, plaque eruption, or cauliflower projection (Patel et al., 2013; Léonard et al., 2014). Its reported prevalence tends to increase. The prevalence of condyloma acuminata in Italian female population during 2009-2010 was 3.8 cases per 1,000 women per year (Suligoi et al., 2017), in the UK population during 2010-2012 was 3.8% in males and 4.6% in females (Sonnenberg et al., 2019), and in the US population during 2013-2014 was 2.9% (Daugherty et al., 2018). A study systematic review on incident of anogenital warts showed 160-289 per

100,000 persons. New incident of anogenital warts was 103-168 per 100,000 persons among males and 76-191 per 100,000 persons among females (Patel et al., 2013).

The most common cause of condyloma acuminata is the infection of Human Papillomavirus (HPV). There are two groups of HPV types, including High risk (HR) HPV and Low risk (LR) HPV. HR-HPV are HPV genotypes 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 61, 73, 82 and LR-HPV are HPV genotypes 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 (Braaten et al., 2008; Gutiérrez-Xicoténcatl et al., 2009). LR-HPV causes condyloma acuminata, but study in China showed that infection of HR-HPV was also found in condyloma acuminata lesions. In some cases, condyloma acuminata is caused by combination infection of LR and HR HPV (Lu et al., 2014). Persistent infection of LR or HR-HPV is a risk factor for transformation of epithelial to be benign hyperplasia or to be premalignant lesions. HR-HPV is related to the occurrence of malignancy in women, as cervical cancer (Santegoets et al., 2012). Some literature states that HR-HPV is considered as the main causative agent responsible for cervical cancer (Braaten et al., 2008). The detection of HPV genotype is very important to do to prevent, establish early diagnosis, and perform therapy in cervical cancer. Determination of LR or HR-HPV in anogenital warts can be used as a factor to predict the progression of lesions to be benign or malignant lesions.

The E7 HPV protein plays a role in the cell transformation process. HPV E7 protein binds to important proteins that play a role in the cell cycle such as the pRB and cyclin A/CDK2 complex that inhibits the interaction between Rb and E2F. The E7 protein of HR-HPV will deactivate pRB resulting in accumulation of $p16^{INK4A}$ protein. The expression of $p16^{INK4A}$ can also be considered as a marker of E7 gene activity (Izadi-Mood al., 2012; Romagosa et al., 2011). $p16^{INK4A}$ plays a role in cell cycle regulation, and is involved in the processes of apoptosis, angiogenesis, cell invasion, and this activity may be associated with overexpression in cancer (Romagosa et al., 2011). The expression of $p16^{INK4A}$ is a marker to determine the prognosis of

a malignancy caused by HPV infection (Missaoui et al., 2010). This suggests that $p16^{INK4A}$ is a specific marker for HPV infection and may correlate with the type of HR-HPV or LR-HPV. The objective was to analyze the correlation between $p16^{INK4A}$ expression and the LR-HPV or HR-HPV in condyloma acuminata lesions. This study identified the genotype of HPV and performed immunohistochemistry (IHC) staining of $p16^{INK4A}$ expression.

Materials and Methods

The samples collection

A cross-sectional study was conducted at Outpatient Clinic of Department Dermatology and Venereology, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, during period January-December 2017. Ethical study was approved by the Medical Ethic Research from Dr. Soetomo General Academic Hospital Surabaya, number 382/Panke.KKE/V/2016.

The sample inclusion criteria were all patients with condyloma acuminata, both male and female, with a clinical diagnosis of condyloma acuminata and willing to participate in this study by signing the informed consent. The sample exclusion criteria were women who were menstruating and / or pregnant, suffering from an active pelvis and / or acute cervicitis, and men or women with a diagnosis of HIV and AIDS, and someone who was not willing to participate in this study.

The specimens were 33 tissues of condyloma acuminata. Tissue from each patient was divided into 2 parts, one part for tissue processing into paraffin block preparations followed by histopathological diagnosis and immuno-histochemistry (IHC) staining, while another for the examination of the HPV genotype. Histopathological diagnose of condyloma acuminata and analysis of IHC staining was performed by pathologist.

The expression of p16^{INK4A}

The expression of p16^{INK4A} was detected by immunohistochemistry (IHC) staining using Anti-CDKN2A/ p16^{INK4A} Antibody (clone 1E12E10) IHC-plus™ LS-B5261 (LS Bio). The interpretation was positive if cells were stained in the nucleus or combined in the nucleus and cytoplasm. The assessment was carried out as previously reported in Klaes et al. (2001). Score 0 (negative) if cells were stained positive <1% of all cells, score 1 (sporadic) if cells were stained positive <5% of all cells, score 2 (focal) if cells wee stained positive <25% of all cells, and the score 3 (diffuses) if the cells were stained positive > 25% of all cells.

The genotyping of HPV

Virus extraction was carried out from condyloma acuminata tissues using the QIAamp DNA Mini Kit (Qiagen) kit, according to the protocol kit. Genotyping of HPV was performed by Polymerase Chain Reaction (PCR), then followed by reverse line blot using the Ampliquality HPV type express v 3.0 kit (Ab Analitica). This kit can detect 40 genotypes of HPV, including HR-HPV were HPV 16, 18, 26, 31, 33,35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68 (68a, 68b), 69, 73, 82 and LR-HPV were HPV 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 64, 70, 71, 72, 81, 83, 84, 87, 89, 90.

Statistical analysis

The difference expression of p16^{INK4A} on condyloma acuminata patients with HR-HPV or LR-HPV groups infection was analyzed by Mann Whitney test (significant if $p < 0.05$). The correlation between variables was analyzed by two-tailed Spearman's rho (significant if $p < 0.05$).

Results

Characteristics of patient

This study was performed on 33 condyloma acuminata patients. Characteristic of patients consisted of sex, age, sexual partner, duration symptom, history, lesion on the partner, therapy, shape, type, and location of lesion. Patients consisted of 12 (36.4%) males and 21 (63.6%) females, ranging from 18 to 64 years, with the highest frequency was 15-24 years by 17/33 (51.5%) persons. Based on sexual partner, it was dominated by heterosexual (men having sex with women) for 30/33 (90.9%). For bisexual (having sex with the same and different sex), it was 2/33 (6.1%) and homosexual (having sex with the same sex) was 1/33 (3.0%). Based on symptom duration, history, lesion on the partner, and therapy, the highest frequency was lesion 1-3 months, the first-time lesion, no lesion on their partner, and received TCA therapy, respectively. Based on the shape, type, and location of lesion, the highest frequency was papule shape, multiple type of lesions, and were in gland penis for male and labia majora for female (Table1).

HPV genotype in condyloma acuminata lesions

The genotype of HPV included infection of HPV HR and LR, both single and multiple infections. The single or multiple infections of HPV LR was assumed as infection of LR- HPV. The multiple infection of LR-HPV with HR-HPV was assumed as infection of HR-HPV. The genotype of LR-HPV were HPV 6, 11, 42, 61, 54, 81, 87, 89, 90 and HR-HPV were 18, 26, 45, 51, 52, 67, 68B, 69, 82. LR-HPV was found in 19/33 (57.6%) patients and HR-HPV were 14/33 (42.4%) patients (Table 2). The most dominant HPV was HPV 11 that infected 24/56 (42.9%) times, then followed by HPV 6 (16.1%) and HPV 18, HPV 51, and HPV 82 for 5.4%, respectively.

$p16^{INK4A}$ expression in condyloma acuminata lesions

The expression of $p16^{INK4A}$ in condyloma acuminata lesions showed that nucleus or the combination of nucleus and cytoplasm of cell was stained in brown color, that indicating in sporadic, focal, or diffuse (Figure 1). The results showed that 7/33 (21.2%) lesion were negative, 13/33 (39.4%) lesions were sporadic, 8/33 (24.2%) lesions were focal, and 5/33 (15.2%) were diffuse. In LR-HPV group, the results showed that negative, sporadic, and focal were 7/19 (36.8%), 9/19 (47.4%), and 3/19 (15.8%), respectively. In HR-HPV group showed that all lesions expressed $p16^{INK4A}$, in sporadic was 4/14 (28.6%), in focal was 5/14 (35.7%), and in diffuse was 5/14 (35.7%) (Table 3). There was a significant different of $p16^{INK4A}$ expression in condyloma acuminata lesion between LR-HPV and HR-HPV groups (p = 0,000). Correlation between $p16^{INK4A}$ expression with LR-HPV and HR-HPV showed moderate correlation (r = 0,644, p = 0,000).

Discussion

The microscopic feature of condyloma acuminata lesions by haematoxylin eosin (HE) staining appear as parakeratosis, hyperkeratosis, hypergranulosis, basal cell hyperplasia, and koilocytic (Léonard et al., 2014). In this study, condyloma acuminata lesions were seen as papules, cauli flowers, and flat-tapping papules. The microscopic feature shown are hyperkeratosis, parakeratosis, papillomatosis, hypergranulosis, and hyperplasia of basal cell, as well as the koilocytes that usually accompany HPV infection.

The most common causes of condyloma acuminata is infection by HPV. There are more than 40 genotypes of HPV that can infect in the anogenital area that are usually infected by LR-HPV

171 including HPV 6 and HPV 11 in single infection, but most commonly co-infection with LR-
172 HPV or HR-HPV (Léonard et al., 2014; Hasanzadeh et al., 2019). This study found all tissues
173 from patients clinically diagnosed as condyloma acuminata were positive for LR-HPV or HR-
174 HPV, in single infection or multiple infection. Most of patients were infected by LR-HPV,
175 while 42.4% were infected by LR-HPV that were co-infected by HR-HPV. This study is in
176 accordance with cross-sectional study in Kuwait, from 156 patients with genital warts showed
177 that 102/156 (65.4%) patents were infected by LR-HPV and 54/156 (34.6%) patients were
178 infected by HR-HPV, in single infection of 88.4% and multiple infection of 11.6% (Al-Awadhi
179 et al., 2019). Study in Spain showed that LR-HPV were 63/138 (45.6%) and 71/138 (41.4%)
180 anogenital warts patients were infected by HR-HPV (Arroyo et al., 2016). Another study found
181 that from 66 anogenital warts specimens, it showed that LR-HPV infected 42/66 (62.1%) which
182 was dominated by HPV 6 (47%), and HPV-11 (13.6%), as well as HPV 18 and HPV 3
183 (Ozaydin-Yavuz et al., 2019). This study indicates that beside LR-HPV, condyloma acuminata
184 is also co-infected with HR-HPV that can develop malignant cancer. Therefore, identification
185 of HPV genotype can predict the risk of developing the diseases. In addition, determination of
186 genotype of HPV has an impact on treatment management, follow-up the outcome of diseases,
187 and prevention strategies

188 This study obtained LR-HPV and also HR-HPV, which 57.6% of condyloma acuminata
189 patients were infected by LR-HPV and the rest were infected by multiple infection of LR-HPV
190 and HR-HPV infection. The most common of HPV genotypes were HPV 11 and HPV 6 for
191 LR-HPV, followed by HPV 18, HPV 51, and HPV 82 for HR-HPV. The incidence of
192 condyloma acuminata and the malignant progression can be prevented by vaccination.
193 Recently, there are 3 commercial vaccines against HPV infection that Gardasil prevents to
194 infection of HPV 6, 11, 16, 18; Cervarix prevents to infection of HPV 16, 18, and Gardasil 9
195 prevent to infection HPV 6, 11, 61, 18, 31, 33, 45, 52, 58 (Gupta et al., 2017). The previous

study on benign lesion of anogenital showed HPV genotype in condyloma acuminata patients was dominated by HPV 11 and HPV 6 from 13 female patients (Arista et al., 2019) and from 12 male patients (Murtiastutik et al., 2019). In pre-cancerous lesion and cancerous lesion of uterine cervix, those were dominated by HPV 16 (62.68%), then followed by HPV 18 (20.9%), HPV 45 (5.97%), 52 (5.97%), and 67 (4.48%) (Mastutik et al., 2018). Vaccination programs is expected to reduce the incidence of these diseases, but there were found some genotype of HPV that could not covered by the current vaccine. Therefore, strategies to prevent the incidence of condyloma acuminata or progression of malignancy still need to be developed.

Oncoprotein E6 and E7 of HR-HPV play a role in cancer development. HR-HPV E6 mediates p53 inactivation by binding to the conserved domains of E6AP (E6-linked protein) to form the E6/ E6AP/p53 complex. This complex causes degradation of p53 by ubiquitination mechanism. HR-HPV E7 targets to degrade the retinoblastoma protein (pRB). In normal cells, when cells are prevented from entering the S phase, the pRB binds to the E2F family of transcription factor, so that the cell stops at the checkpoint of G1-S phase, activating cell cycles arrest. pRb which is phosphorylated by cyclin D1/CDK 4/6 complex causes E2F released and enter the nucleus. The cell enters the S phase which then starts the activation of gene transcription (Munger et al., 2013, Pal and Kudu, 2020). Furthermore, phosphorylated pRB is a $p16^{INK4A}$ feedback mechanism. HPV E7 induces degradation of pRB by a ubiquitin proteosome pathway that causes the loss feedback mechanism of $p16^{\mathbf{INK4A}}$ and leads to accumulation of $p16^{\mathbf{INK4A}}$ that presents as overexpression of $p16^{\mathbf{INK4A}}$ (Lassen et al., 2009; Faraji et al., 2017).

The overexpression of $p16^{INK4A}$ in this study was sporadic (39.4 %), focal (24.2%), diffuse (15.2%), dan negative (21.2%). Another study of $p16^{INK4A}$ expression in 24 condyloma acuminata specimens showed variable in which 11/24 (45.8%) were sporadic, 7/24 (29.2%) were focal, and 6/24 (25%) were negative (Kazlouskaya et al., 2013). The expression of $p16^{INK4A}$ in cervix tended to increase from cervical normal epithelium to invasive cervical

cancer (Missaoui et al., 2010; Izadi-Mood et al., 2012) and cervical adenocarcinoma (Mastutik et al., 2021). The expression of $p16^{INK4A}$ in HR-HPV showed all specimens were positive ranging from sporadic to diffuse. In LR-HPV were sporadic and focal, whereas 36.8 % of specimens were not expressed in $p16^{\mathbf{INK4A}}$ and none of specimens expressed in diffuse category. Other study found in anal lesion with HR HPV infection showed all specimens expressed $p16^{INK4A}$ in ranging from sporadic to diffuse, whereas those infected with LR-HPV were mostly patchy (Leeman et al., 2019), in oropharyngeal squamous cell carcinomas and tonsillar dysplasia that was positive for HPV 16, was also diffuse, but in benign and pre-malignant lesions were positive for HPV 6 and HPV 11 showed variations from negative to strong positive (Mooren et al., 2014). All cervical lesions with HR-HPV had a significant $p16^{INK4A}$ expression with a strong and diffuse expression, whereas that with LR-HPV showed mild expression (score 1) (Missaoui et al., 2014).

The finding highlight from this study is the expression of $p16^{INK4A}$ significantly correlated with HR-HPV infection in which condyloma acuminata lesions infected by HR-HPV have possibility 0.644 times to express $p16^{INK4A}$ compared to condyloma acuminata lesions infected by LR-HPV. As previous studies showed that $p16^{INK4A}$ expression concordance with HR-HPV infection in oropharyngeal squamous (Liu et al., 2015), in mucosal squamous cell carcinomas of the head and neck (Antonsson et al., 2015), in cervical squamous intraepithelial lesion (Yildiz et al., 2007), and invasive cervical carcinoma and vagina (Missaoui et al., 2010; Missaoui et al., 2014). HR-HPV is integrated into the host cell genome, whereas LR-HPV prefers extra chromosome as episome so that the expression of E6 and E7 oncoproteins are within the regulatory framework of E1 and E2 HPV (Boulet et al., 2007). HR-HPV, such as HPV 18, 33, HPV 51, HPV 58, HPV 59 was found to be integrated, while HPV 30, 35, 39, 44, 45, 53, 56, 59, 74 and 82 were not integrated but were in episome form (Nkili-Meyong et al., 2019). In this study found HR-HPV 18, 51 and 82. As reported in Nkili-Meyong et al. (2019) that HPV 18

was founded to be integrated in 55% of the positive HPV 18 specimen, and HPV 51 was integrated in 25% of the positive HPV 51 specimen, and HPV 82 was founded in extra chromosome. This integration was associated with partial or total deletion of E1 and E2 gene, leading to overexpression of E6 and E7 due to loss feedback mechanism by E2 protein (Woodman et al., 2007; Nkili-Meyong et al., 2019). In addition, the HR-HPV E7 oncoproteins have the higher affinity to bind pRB than LR-HPV E7 that increase the accumulation of $p16^{INK4A}$. Therefore, all specimens infected by HR-HPV in this study showed positive for the expression of $p16^{INK4A}$ showing in sporadic, focal, and diffuse, while LR-HPV showed in sporadic and focal, and no diffuse expression.

In conclusion, this study found significant differences between expression of $p16^{INK4A}$ in condyloma acuminata infected by LR-HPV (HPV 6, 11, 42, 61, 54, 81, 87, 89, 90) and HR-HPV (HPV 18, 26, 45, 51, 52, 67, 68B, 69, 82). This $p16^{INK4A}$ expression is correlated with HR-HPV infection in moderate. IHC is a routine method to perform the diagnostic of histopathological. Therefore, IHC of $p16^{INK4A}$ could be used as biomarker for HR-HPV infection that might be useful to predict the malignancy development of condyloma acuminata lesions.

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Author's contribution:

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- Clinical studies: Gondo Mastutik, Dwi Murtiastutik, Trisniartami Setyaningrum
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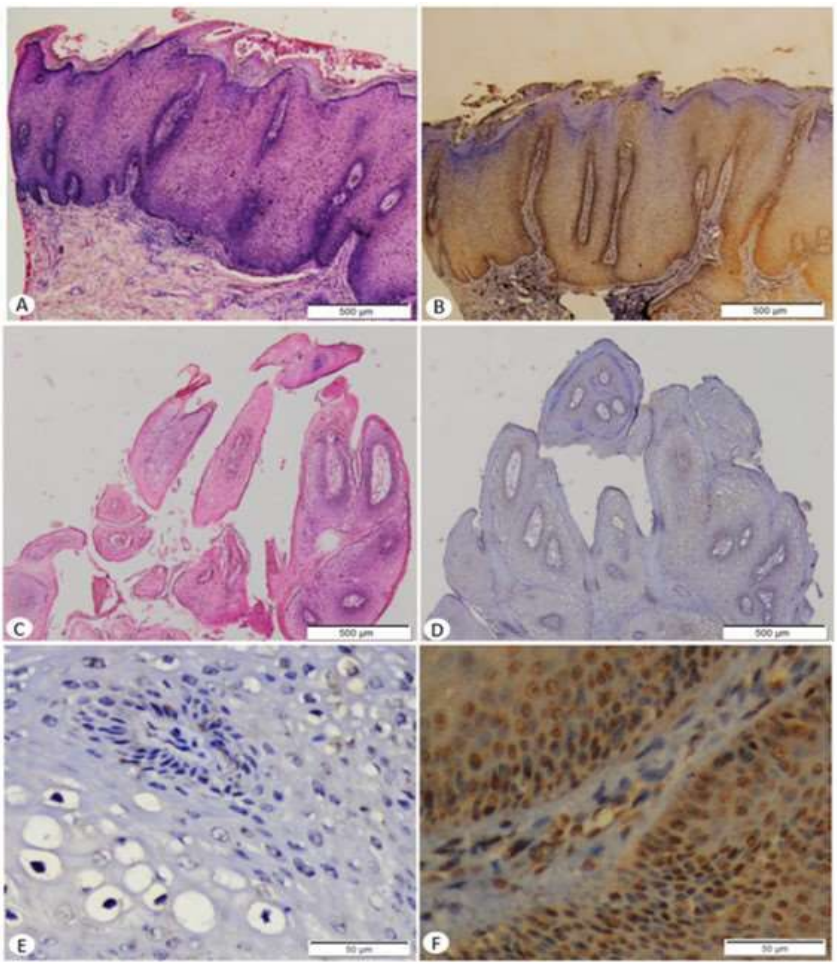

Figure 1. The histopathological feature and $p16^{INK4A}$ expression of condyloma acuminata. A) Condyloma acuminata from HR-HPV infected patients, in HE staining (40 x magnification); B) In the same patients, $p16^{INK4A}$ expression showed in diffuse (score 3) (40 x magnification); C) Condyloma acuminata from LR-HPV infected patients, in HE staining (40 x magnification); D) In the same patients, $p16^{INK4A}$ expression showed negative (score 0) (40 x magnification); E) $p16^{INK4A}$ expression in negative (score 0) (400 x magnification); F) $p16^{INK4A}$ expression in diffuse (score 3) (400 x magnification).

409 Table 1. Characteristics of patients with condyloma acuminata

Characteristics of Patients	N (%)	Single or multiple infection LR/LR HPV N (%)	Multiple infection of LR/HR HPV N (%)
Sex:			
- Male	12 (36.4)	7 (21.2)	5 (15.1)
- Female	21 (63.6)	12 (36.4)	9 (27.3)
Age:			
- 15-24 years	17 (51,5)	10 (30.3)	7 (21.2)
- 25-34 years	6 (18,2)	3 (9.1)	3 (9.1)
- 35-44 years	7 (21,2)	4 (12.1)	3 (9.1)
- 45-54 years	2 (6,1)	1 (3.0)	1 (3.0)
- 55-64 years	1 (3,0)	1 (3.0)	0
Sexual Partner			
- Heterosexuals	30 (90,9)	18 (54.5)	12 (36.4)
- Homosexual	1 (3,0)	1 (3.0)	0
- Bisexual	2 (6,1)	0	2 (6.1)
Duration of symptom			
- < 1 month	5 (15,1)	1 (3.0)	4 (12.1)
- 1- 3 months	20 (61,6)	14 (42.4)	6 (18.2)
- 4 - 6 months	6 (18,2)	3 (9.1)	3 (9.1)
- > 6 months	2 (6,1)	1 (3.0)	1 (3.0)
History			
- First lesion	26 (78,8)	15 (45.4)	11 (33.3)
- Recurrent lesion	7 (21,2)	4 (12.1)	3 (9.1)
Lesion on the partner			
- Have lesion on the partner	4 (12,1)	2 (6.1)	2 (6.1)
- No lesions on the partner	29 (87,9)	17 (51,5)	12 (36.4)
Therapy			
- TCA	19 (57,6)	11 (33.3)	8 (24.2)
- Cautery	14 (42,4)	8 (24.2)	6 (18.2)
Shape			
- Papule	28 (84,8)	16 (48.5)	12 (36.4)
- Cauli flower	4 (12,1)	2 (6.1)	2 (6.1)
- Flat tapped papule	1 (3,0)	1 (3.0)	0
Type			
- Multiple	31 (93,9)	18 (54.5)	13 (39.4)
- Solitary	2 (6,1)	1 (3.0)	1 (3.0)
Location			
- Penile	7 (21,2)	4 (12.1)	3 (9.1)
- Anus	4 (12,1)	2 (6.1)	2 (6.1)
- Penile and anus	1 (3.0)	1 (3.0)	0
- Labia (majora and minora)	16 (48,5)	10	6 (18.2)
- Vulva	3 (9,1)	1 (3.0)	2 (6.1)
- Introitus vagina	1 (3.0)	0	1 (3.0)
- Perineum	1 (3.0)	1 (3.0)	0

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411 Table 2. Distribution of HPV in condyloma acuminata lesions

Genotype of HPV	HR or LR HPV genotypes	Frequency N (%)
LR HPV		19 (57,6)
- HPV 6	LR	4
- HPV 11	LR	12
- HPV 6, 11	LR	2
- HPV 6,81,87,89	LR	1
HR HPV		14 (42,4)
- HPV 6,11,18,51,82	HR	1
- HPV 6,42,51,61	HR	1
- HPV 11, 18	HR	2
- HPV 11,18,45	HR	1
- HPV 11, 26	HR	1
- HPV 11,67	HR	1
- HPV 11, 51, 82	HR	1
- HPV 11, 52, 54	HR	1
- HPV 11, 52,69,90	HR	1
- HPV 11, 68B	HR	1
- HPV 11, 82	HR	2
Total		33

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426 Table 3. The p16 ^{INK4A} expression on condyloma acuminata that infected by LR or HR-HPV

p16 ^{INK4A} expression	HPV genotype		Total	p-value
	LR-HPV N (%)	LR/HR-HPV N (%)	N (%)	
Score 0 (Negative)	7 (36,8%)	0 (0%)	7 (21,2%)	0,000*
Score 1 (Sporadic)	9 (47,4%)	4 (28,6%)	13 (39,4%)	
Score 2 (Focal)	3 (15,8%)	5 (35,7%)	8 (24,2%)	
Score 3 (Diffuse)	0 (0%)	5 (35,7%)	5 (15,2%)	
Total	19 (100%)	14 (100%)	33 (100%)	

*Mann Whitney, Asymptotic Significance (2-tailed) with p = 0,000 (p < 0,05) Correlation is significant at the 0.01 level (2-tailed) with Spearman's rho (r) = 0,644, p = 0,000)

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Commented [A1]:

Before revision & after revision based on reviewer comments

Reviewer-commented-APJCP-2105-6861 edit GM

Before and after

Comment	Before	After
Major comment: the study includes HPV that is interest to cancer science. However, the English writing is very poor. Needs serious overhauling.		
Abtract		
Wrong sentence. It should read: A cross-sectional study was conducted during January-December 2017 on 33 condyloma acuminata tissues sample. The study was approved by ethical commission ….. No need of ethical clearance in the abstract. Not namely, you do not name you score. English literacy problem. No need to bring statistical analysis in the abstract.	**Objective:** The objective was to analyze the correlation between $p16^{INK4A}$ expression and the LR-HPV and HR-HPV infection in condyloma acuminata lesions. **Methods:** A cross-sectional study was conducted during January-December 2017 on 33 condyloma acuminata tissues which has been approved by ethical commission. The expression of $p16^{INK4A}$ was detected by immunohistochemistry (IHC) staining. The positive interpretation was carried out by giving a score, namely score 0 (negative), score 1 (sporadic), score 2 (focal) and	**Objective:** The objective was to analyze the correlation between $p16^{INK4A}$ expression and the LR/HR-HPV infection in condyloma acuminata lesions. **Methods:** A cross-sectional study was conducted during January-December 2017 on 33 condyloma acuminata patients. The expression of $p16^{INK4A}$ was detected by immunohistochemistry (IHC) staining. The positive interpretation was carried out by scoring which score 0 was negative, score 1 was sporadic, score 2 was focal, and score 3 was diffuses. The HPV genotypes

	score 3 (diffuses). The 40 genotypes of HPV were detected by Polymerase Chain Reaction and followed by reverse line blot. The $p16^{INK4A}$ expression data were tested by Mann Whitney, and correlation between variables was analyzed by two-tailed Spearman's rho.	were identified by reverse line blot that detected 40 genotypes of HPV, including HR-HPV (HPV 16, 18, 26, 31, 33,35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68a, 68b, 69, 73, 82) and LR-HPV (HPV 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 64, 70, 71, 72, 81, 83, 84, 87, 89, 90).
Define the two group in the method section of the abstract. Needs English editing.	**Results:** The $p16^{INK4A}$ expression between LR-HPV and HR-HPV groups was significantly different (p = 0,000) and showed the moderate correlation (r = 0,644). LR-HPV in condyloma acuminata were HPV 6, 11, 42, 61, 54, 81, 87, 89, 90 and HR-HPV 18, 26, 45, 51, 52, 67, 68B, 69, 82. LR-HPV was found in 19/33 patients and HR-HPV was in 14/33 patients. In general, $p16^{INK4A}$ expression showed that 15.2% was diffuse, 24.2% lesion was focal, 39.4% lesion was sporadic, and 21.2% lesion was negative.	Mentions in methods **Results:** The expression of $p16^{INK4A}$ was significantly correlated with HR-HPV which patients infected by HR-HPV have possibility 0.644 times to express $p16^{INK4A}$ compared to infected by LR-HPV. LR-HPV in condyloma acuminata were HPV 6, 11, 42, 61, 54, 81, 87, 89, 90 and HR-HPV were HPV 18, 26, 45, 51, 52, 67, 68B, 69, 82. LR-HPV was found in 19/33 patients and HR-HPV was in 14/33 patients. The expression of $p16^{INK4A}$ in condyloma acuminata lesions showed that 15.2% patients were diffuse, 24.2%

Grammar problem, it should read: In the LR-HPV group, there was no…	In LR-HPV group showed that there was no diffuse expression, focal was 15.8%, sporadic was 47.4%, and negative was 36.8%, while in HR-HPV group, it showed that all lesions expressed $p16^{INK4A}$, in diffuse was 35.7%, in focal was 35.7%, and in sporadic was 28.6%.	patients were focal, 39.4% patients were sporadic, and 21.2% patients were negative. In LR-HPV group, there was no diffuse expression, focal was 15.8%, sporadic was 47.4%, and negative was 36.8%, while in HR-HPV group, all lesions expressed $p16^{INK4A}$, in diffuse was 35.7%, in focal was 35.7%, and in sporadic was 28.6%.
Part of result. Conclusion is mostly a qualitative expression of your finding.	**Conclusion:** The most dominant HPV was LR-HPV 11, followed by HPV 6, HPV 18, HPV 51, and HPV 82. The expression of $p16^{INK4A}$ was significantly correlated with HR-HPV infection in which condyloma acuminata lesions infected by HR-HPV have possibility 0.644 times to express $p16^{INK4A}$ compared to infected by LR-HPV.	**Conclusion:** IHC is a routine method in histopathological diagnosis, therefore the detection of p16INK4A expression by IHC can be used as a biomarker for HR-HPV infection.
Introduction		
Wrong word. Wrong sentence in terms of English. English problem	Condyloma acuminata is the most frequent sexually transmitted disease that often occurs worldwide (Santegoets et al., 2012). It is also referred to genital warts or anogenital warts. These lesions are usually in single or	Condyloma acuminata is the most frequent sexually transmitted disease that often occurs worldwide (Santegoets et al., 2012). It is also referred to genital warts or anogenital warts. These lesions are usually in

	multiple lesions in the anogenital region accompanied by symptoms of itching, vaginal discharge, and bleeding. The forms are flat or lobulated that looks pearl-like, filiform, plaque eruption, or cauliflower projection (Patel et al., 2013; Léonard et al., 2014). Its reported prevalence tends to increase. The prevalence of the Italian female population during 2009-2010 was 3.8 cases per 1,000 women per year (Suligoi et al., 2017). In addition, the prevalence in the UK population during 2010-2012 was 3.8% in males and 4.6% in females (Sonnenberg et al., 2019), and in the US population during 2013-2014 was 2.9% (Daugherty et al., 2018). A study systematic review on incident of anogenital warts showed 160-289 per 100,000 persons. New incident of anogenital warts was 103-168 per 100,000 persons among males and 76-191 per 100,000 persons among females (Patel et al., 2013).	single or multiple lesions in the anogenital region accompanied by symptoms of itching, vaginal discharge, and bleeding. The type of shape is flat or lobulated that looks pearl-like, filiform, plaque eruption, or cauliflower projection (Patel et al., 2013; Léonard et al., 2014). Its reported prevalence tends to increase. The prevalence of condyloma acuminata in Italian female population during 2009-2010 was 3.8 cases per 1,000 women per year (Suligoi et al., 2017), in the UK population during 2010-2012 was 3.8% in males and 4.6% in females (Sonnenberg et al., 2019), and in the US population during 2013-2014 was 2.9% (Daugherty et al., 2018). A study systematic review on incident of anogenital warts showed 160-289 per 100,000 persons. New incident of anogenital warts was 103-168 per 100,000 persons among males and 76-191 per 100,000 persons among females (Patel et al., 2013).

	This suggests that $p16^{INK4A}$ is a specific marker for HPV infection and may have a correlation with the type of HR-HPV or LR-HPV. The objective was to analyze the correlation between $p16^{INK4A}$ expression with the LR-HPV and HR-HPV in condyloma acuminata lesions. This study identified the genotype of HPV and performed immunohistochemistry (IHC) staining of $p16^{INK4A}$ expression.	This suggests that $p16^{INK4A}$ is a specific marker for HPV infection and may correlate with the type of HR-HPV or LR-HPV. The objective was to analyze the correlation between $p16^{INK4A}$ expression and the LR-HPV or HR-HPV in condyloma acuminata lesions. This study identified the genotype of HPV and performed immunohistochemistry (IHC) staining of $p16^{INK4A}$ expression.
Materials and Methods		
Wrong sentence	The difference between $p16^{INK4A}$ expression and HR-HPV and LR-HPV genotypes was tested by Mann Whitney test (significant if $p < 0.05$), whereas the correlation between variables was analyzed by two-tailed Spearman's rho (significant if $p < 0.05$).	The difference expression of $p16^{INK4A}$ on condyloma acuminata patients with HR-HPV or LR-HPV groups infection was analyzed by Mann Whitney test (significant if $p < 0.05$). The correlation between variables was analyzed by two-tailed Spearman's rho (significant if $p < 0.05$).
Result		
Be care full that you studied patients not tissue. In fact, the unite of your study was patients with wart. You sample tissue	This study was performed on 33 tissues obtained from condyloma acuminata patients. Characteristic of patients	This study was performed on 33 condyloma acuminata patients. Characteristic of patients consisted of sex, age,

from patient. The English is weak and the expression is not fully understood.	consisted of sex, age, sexual partner, duration symptom, history of lesion, history of lesion on the partner, type of therapy, efflorescence form, type of lesion, and location of lesion. Patients consisted of 12 (36.4%) males and 21 (63.6%) females, ranging from 18 to 64 years, with the highest frequency was 15-24 years by 17/33 (51.5%) persons.	sexual partner, duration symptom, history, lesion on the partner, therapy, shape, type, and location of lesion. Patients consisted of 12 (36.4%) males and 21 (63.6%) females, ranging from 18 to 64 years, with the highest frequency was 15-24 years by 17/33 (51.5%) persons. Based on sexual partner, it was dominated by heterosexual (men having sex with women) for 30/33 (90.9%). For bisexual (having sex with the same and different sex), it was 2/33 (6.1%) and homosexual (having sex with the same sex) was 1/33 (3.0%). Based on symptom duration, history, lesion on the partner, and therapy, the highest frequency was lesion 1-3 months, the first-time lesion, no lesion on their partner, and received TCA therapy, respectively. Based on the shape, type, and location of lesion, the highest frequency was papule shape, multiple type of lesions, and were in gland penis for male and labia majora for female (Table1).

BEFORE

Table 3. The p16 ^{INK4A} expression on condyloma acuminata that infected by LR or HR HPV

p16 ^{INK4A} expression	HPV genotype		Percentage (%)	p-value
	LR-HPV	LR/HR-HPV		
Score 0 (Negative)	7 (36,8%)	0 (0%)	7 (21,2%)	0,000
Score 1 (Sporadic)	9 (47,4%)	4 (28,6%)	13 (39,4%)	
Score 2 (Focal)	3 (15,8%)	5 (35,7%)	8 (24,2%)	
Score 3 (Diffuse)	0 (0%)	5 (35,7%)	5 (15,2%)	
Total	19 (100%)	14 (100%)	33 (100%)	

Commented [A1]:

Comment: Value must be attributed to a comparison. You need to define what comparison is so significant. Seek help from epidemiologist or bio-statisticians.

What is this: what is 7 what is 12, then what is 2%. All must be defined in the column heading.

AFTER

Table 3. The p16 ^{INK4A} expression on condyloma acuminata that infected by LR or HR-HPV

p16 ^{INK4A} expression	HPV genotype		Total N (%)	p-value
	LR-HPV N (%)	LR/HR-HPV N (%)		
Score 0 (Negative)	7 (36,8%)	0 (0%)	7 (21,2%)	0,000*
Score 1 (Sporadic)	9 (47,4%)	4 (28,6%)	13 (39,4%)	
Score 2 (Focal)	3 (15,8%)	5 (35,7%)	8 (24,2%)	
Score 3 (Diffuse)	0 (0%)	5 (35,7%)	5 (15,2%)	
Total	19 (100%)	14 (100%)	33 (100%)	
*Mann Whitney, Asymptotic Significance (2-tailed) with p = 0,000 (p < 0,05) Correlation is significant at the 0.01 level (2-tailed) with Spearman's rho (r) = 0,644, p = 0,000)				

Commented [A2]:

Table 1. Characteristics of patients with Condyloma Acuminata

Characteristics of Patients	N (%)	Single or multiple infection LR/LR HPV N (%)	Multiple infection of LR/HR HPV N (%)
Sex:			
- Male	12 (36.4)	7 (21.2)	5 (15.1)
- Female	21 (63.6)	12 (36.4)	9 (27.3)
Age:			
- 15-24 years	17 (51,5)	10 (30.3)	7 (21.2)
- 25-34 years	6 (18,2)	3 (9.1)	3 (9.1)
- 35-44 years	7 (21,2)	4 (12.1)	3 (9.1)
- 45-54 years	2 (6,1)	1 (3.0)	1 (3.0)
- 55-64 years	1 (3,0)	1 (3.0)	0
Sexual Partner			
- Heterosexuals	30 (90,9)	18 (54.5)	12 (36.4)
- Homosexual	1 (3,0)	1 (3.0)	0
- Bisexual	2 (6,1)	0	2 (6.1)
Duration of symptom			
- < 1 month	5 (15,1)	1 (3.0)	4 (12.1)
- 1- 3 months	20 (61,6)	14 (42.4)	6 (18.2)
- 4 - 6 months	6 (18,2)	3 (9.1)	3 (9.1)
- > 6 months	2 (6,1)	1 (3.0)	1 (3.0)
History			
- First lesion	26 (78,8)	15 (45.4)	11 (33.3)
- Recurrent lesion	7 (21,2)	4 (12.1)	3 (9.1)
Lesion on the partner			
- Have lesion on the partner	4 (12,1)	2 (6.1)	2 (6.1)
- No lesions on the partner	29 (87,9)	17 (51,5)	12 (36.4)
Therapy			
- TCA	19 (57,6)	11 (33.3)	8 (24.2)
- Cautery	14 (42,4)	8 (24.2)	6 (18.2)
Shape			
- Papule	28 (84,8)	16 (48.5)	12 (36.4)
- Cauli flower	4 (12,1)	2 (6.1)	2 (6.1)
- Flat tapped papule	1 (3,0)	1 (3.0)	0
Type			
- Multiple	31 (93,9)	18 (54.5)	13 (39.4)
- Solitary	2 (6,1)	1 (3.0)	1 (3.0)
Location			
- Penile	7 (21,2)	4 (12.1)	3 (9.1)
- Anus	4 (12,1)	2 (6.1)	2 (6.1)
- Penile and anus	1 (3.0)	1 (3.0)	0
- Labia (majora and minora)	16 (48,5)	10	6 (18.2)
- Vulva	3 (9,1)	1 (3.0)	2 (6.1)
- Introitus vagina	1 (3.0)	0	1 (3.0)
- Perineum	1 (3.0)	1 (3.0)	0

Table 2. Distribution of HPV in condyloma acuminata lesions

Genotype of HPV	**HR or LR HPV genotypes**	**Frequency N (%)**
LR HPV		19 (57,6)
- HPV 6	LR	4
- HPV 11	LR	12
- HPV 6, 11	LR	2
- HPV 6,81,87,89	LR	1
HR HPV		14 (42,4)
- HPV 6,11,18,51,82	HR	1
- HPV 6,42,51,61	HR	1
- HPV 11, 18	HR	2
- HPV 11,18,45	HR	1
- HPV 11, 26	HR	1
- HPV 11,67	HR	1
- HPV 11, 51, 82	HR	1
- HPV 11, 52, 54	HR	1
- HPV 11, 52,69,90	HR	1
- HPV 11, 68B	HR	1
- HPV 11, 82	HR	2
Total		33

RESEARCH ARTICLE

Editorial Process: Submission:00/00/0000 Acceptance:00/00/0000

p16^{INK4A} Expression in Condyloma Acuminata Lesions Associated with High-Risk Human Papillomavirus Infection

Gondo Mastutik[1]*, Alphania Rahniayu[1,2], Afria Arista[2], Dwi Murtiastutik[1,2], Nila Kurniasari[1,2], Trisniartami Setyaningrum[1,2], Anny Setijo Rahaju[1,2]

Abstract

Objective: The objective of this study was to discover the possible correlation between p16INK4A expression and the LR/HR-HPV infection in condyloma acuminate (CA) lesions. **Materials and Method:** This cross-sectional study was conducted during January-December 2017 on 33 CA patients. The expression of p16^{INK4A} was detected by immunohistochemistry (IHC) staining. The positive interpretation was carried out by scoring which score 0 was negative, score 1 was sporadic, score 2 was focal, and score 3 was diffuses. The HPV genotypes were identified by reverse line blot, and 40 genotypes of HPV detected, including HR-HPV (HPVs 16, 18, 26, 31, 33,35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68a, 68b, 69, 73, and 82) and LR-HPV (HPVs 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 64, 70, 71, 72, 81, 83, 84, 87, 89, and 90). **Results:** The expression of p16^{INK4A} was significantly correlated with HR-HPV infection. Patients infected with HR-HPV had 0.644 times higher possibility to express p16^{INK4A} gene compared to those infected with LR-HPV. LR-HPV genotypes detected in CA patients were HPVs 6, 11, 42, 61, 54, 81, 87, 89, and 90 and HR-HPV genotypes were HPVs 18, 26, 45, 51, 52, 67, 68B, 69, and 82. LR-HPV was found in 19/33 of patients and HR-HPV was in 14/33 of patients. The expression of p16^{INK4A} in CA lesions was diffuse in15.2% of patients, was focal in 24.2% of patients , was sporadic in 39.4% of patients were, and was negative in 21.2% of patients . In LR-HPV group, there was no diffuse expression, focal expression was observed in 15.8%, sporadic in 47.4%, and negative in 36.8%, while in HR-HPV group, p16^{INK4A} expression was detected in all lesions , in a way that its expression was diffuse in 35.7%, focal in 35.7%, and sporadic in 28.6%. **Conclusion:** IHC is a routine method in histopathological diagnosis, therefore the detection of p16^{INK4A} expression by IHC can be used as a biomarker for HR-HPV infection diagnosis.

Keywords: p16^{INK4A}- high-risk HPV- low-risk HPV- condyloma- sexually transmitted diseases

Asian Pac J Cancer Prev, **22**,

Introduction

Condyloma acuminata (CA) is the most frequent sexually transmitted disease worldwide (Santegoets et al., 2012). It is also known as genital warts or anogenital warts. These lesions are usually single or multiple appearing in the anogenital region and causing symptoms of itching, vaginal discharge, and bleeding. CA can be flat or lobulated and appears as pearl-like, filiform, plaque eruption, or cauliflower projection (Patel et al., 2013; Léonard et al., 2014). Its prevalence is increasing worldwide. The prevalence of CA in Italian female population during 2009-2010 was 3.8 cases per 1,000 women per year (Suligoi et al., 2017), in the UK population during 2010-2012 was 3.8% in males and 4.6% in females (Sonnenberg et al., 2019), and in the US population during 2013-2014 was 2.9% (Daugherty et al., 2018). A systematic review on the incident of anogenital warts showed that their prevalence ranged from 160 to 289 per 100,000 persons. New incident of anogenital warts ranged from 103 to 168 per 100,000 persons among males and 76 to 191 per 100,000 persons among females (Patel et al., 2013).

The most common cause of CA is Human Papillomavirus (HPV). There are two groups of HPV, including high risk (HR) HPV and low risk (LR) HPV. HR-HPV are HPV genotypes of 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 61, 73, and 82, and LR-HPV are HPV genotypes of 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81 (Braaten et al., 2008; Gutiérrez-Xicoténcatl et al., 2009). LR-HPV causes CA, but a study in China showed that the infection of HR-HPV was also found in CA lesions. In some cases, CA is caused by the combination of LR and HR-HPV infections (Lu et al., 2014). Persistent infection of LR or HR-HPV is a risk factor for the transformation of epithelial to benign hyperplasia or

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premalignant lesions. HR-HPV is related to the occurrence of malignancy in women, as cervical cancer (Santegoets et al., 2012). Some literature considers HR-HPV as the main causative agent responsible for the cervical cancer (Braaten et al., 2008). The detection of HPV genotype is very important to prevent, establish early diagnosis, and initiate the treatment in cervical cancer. Determination of LR or HR-HPV in anogenital warts can be used as a factor to predict the progression of lesions to benign or malignant lesions.

The E7 HPV protein plays a role in the cell transformation process. It binds to important proteins, such as the pRB and cyclin A/CDK2 complex, inhibiting the interaction between Rb and E2F. The E7 protein of HR-HPV deactivates pRB, resulting in the accumulation of $p16^{INK4A}$ protein. The expression of $p16^{INK4A}$ can also be considered as a marker of E7 gene activity (Izadi-Mood al., 2012; Romagosa et al., 2011). $p16^{INK4A}$ plays a role in cell cycle regulation and it is involved in the processes of apoptosis, angiogenesis, cell invasion. This activity may be associated with overexpression in cancer (Romagosa et al., 2011). The expression of $p16^{INK4A}$ is a marker to determine the prognosis of a malignancy caused by HPV infection (Missaoui et al., 2010), suggesting that $p16^{INK4A}$ can be a specific marker for HPV infection and may correlate with the type of HR-HPV or LR-HPV. The objective of this study was to analyze the correlation between $p16^{INK4A}$ expression and the LR-HPV or HR-HPV in CA lesions. This study identified the genotype of HPV and performed immunohistochemical analysis of $p16^{INK4A}$ staining of.

Materials and Methods

The samples collection

This cross-sectional study was conducted at Outpatient Clinic of Department Dermatology and Venereology, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, from January 2017 to December 2017. The study was ethically approved by the Medical Ethic Research at Dr. Soetomo General Academic Hospital, Surabaya (ethical code: 382/Panke.KKE/V/2016).

All patients with CA, both male and female, who were willing to participate in the study, were included. Informed consent was obtained from the participants. Menstruating and pregnant women, those who suffered from an active pelvis and / or acute cervicitis, men or women with a diagnosis of HIV and AIDS, and those who were not willing to participate in the study were excluded.

The specimens were 33 tissues of CA. Tissue from each patient was divided into 2 parts, one part for tissue processing into paraffin block preparations followed by histopathological diagnosis and immunohistochemistry (IHC) staining, while another for the examination of the HPV genotype. Histopathological diagnosis of CA and analysis of IHC staining was performed by a pathologist.

Expression of $p16^{INK4A}$

The expression of p16INK4A was detected by IHC staining using Anti-CDKN2A/ $p16^{INK4A}$ Antibody (clone 1E12E10) IHC-plus™ LS-B5261 (LS Bio). The interpretation was positive if cells were stained in the nucleus or combined in the nucleus and cytoplasm. The assessment was carried out based on guidelines presented by Klaes et al. (2001) as follows:

Score 0 (negative): if cells were stained positive <1% of all cells

Score 1 (sporadic): if cells were stained positive <5% of all cells

Score 2 (focal): if cells wee stained positive <25% of all cells

Score 3 (diffuses): if cells were stained positive > 25% of all cells.

Genotyping of HPV

Virus extraction was carried out from CA tissues using the QIAamp DNA Mini Kit (Qiagen) kit and according to the manufacturer's protocol. Genotyping of HPV was performed by Polymerase Chain Reaction (PCR), then followed by reverse line blot using the Ampliquality HPV type express v 3.0 kit (Ab Analitica). This kit could detect 40 genotypes of HPV, including HR-HPV were HPV 16, 18, 26, 31, 33,35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68 (68a, 68b), 69, 73, and 82 and LR-HPV were HPV 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 64, 70, 71, 72, 81, 83, 84, 87, 89, and 90.

Statistical analysis

The difference in the expression of $p16^{INK4A}$ on CA patients with HR-HPV or LR-HPV groups infection was analyzed by Mann Whitney test (significant if $p < 0.05$). The correlation between variables was analyzed by two-tailed Spearman's rho (significant if $p < 0.05$).

Results

Characteristics of patient

This study was performed on 33 patients suffering from CA. Patients' characteristic, such as sex, age, sexual partner, duration of the symptoms, medical history, presence of lesion in the partner, history of treatment, and shape, type, and location of the lesion were investigated. The patients consisted of 12 (36.4%) males and 21 (63.6%) females. The patients aged from 18 to 64 years. The highest frequency was allocated to the age group of 15-24 years old (51.5%). Regarding sexual partner, it was found that 30/33 of the patients (90.9%) were heterosexual (men having sex with women) , 2/33 (6.1%) were bisexual (having sex with the same and different sex), and 1/33 (3.0%) were homosexual (having sex with the same sex) . Regarding symptoms duration, the highest frequency was allocated to the duration of 1to 3 months. Mostly, the patients experienced CA for the first time, there was no lesion in their partner, and received TCA therapy, respectively. Considering the shape, type, and location of the lesion, it was revealed that the highest frequencis were allocated to papule shape, multiple type of lesions, and gland penis for male and labia majora for female (Table1).

HPV genotype in CA lesions

The genotype of HPV included the infection of HPV-

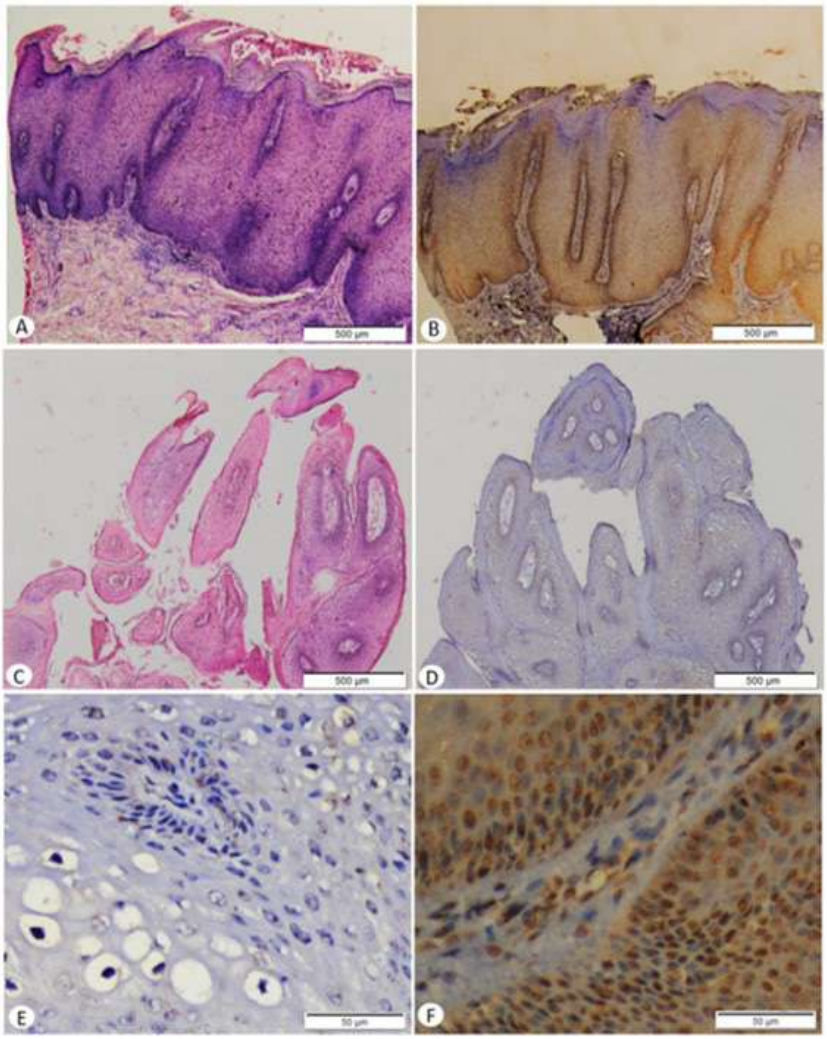

Figure 1. The Histopathological Feature and p16^{INK4A} Expression of Condyloma Acuminata. A, Condyloma acuminata from HR-HPV infected patients, in HE staining (40 x magnification); B, In the same patients, p16^{INK4A} expression showed in diffuse (score 3) (40 x magnification); C, Condyloma acuminata from LR-HPV infected patients, in HE staining (40 x magnification); D, In the same patients, p16INK4A expression showed negative (score 0) (40 x magnification); E, p16^{INK4A} expression in negative (score 0) (400 x magnification); F, p16^{INK4A} expression in diffuse (score 3) (400 x magnification).

HR and LR, both single and multiple infections. The single or multiple infections of HPV-LR was assumed as infection of LR- HPV. The multiple infection of LR-HPV with HR-HPV was assumed as infection of HR-HPV. The genotype of LR-HPV were HPV 6, 11, 42, 61, 54, 81, 87, 89, and 90, and of HR-HPV were 18, 26, 45, 51, 52, 67, 68B, 69, and 82. LR-HPV was found in 19/33 (57.6%) of the patients and HR-HPV was seen in 14/33 (42.4%) of the patients (Table 2). The most dominant HPV was HPV 11 that infected 24/56 (42.9%) times more, then followed by HPV 6 (16.1%) and HPV 18, HPV 51, and HPV 82 for 5.4%, respectively.

p16^{INK4A} expression in CA lesions

The expression of p16^{INK4A} in CA lesions showed that nucleus or the combination of nucleus and cytoplasm of cell was stained in brown color, indicating in sporadic, focal, or diffuse (Figure 1). The results showed that 7/33 (21.2%) of the lesions were negative, 13/33 (39.4%) were sporadic, 8/33 (24.2%) were focal, and 5/33 (15.2%) were diffuse. In LR-HPV group, the results showed that negative, sporadic, and focal lesions were 7/19 (36.8%), 9/19 (47.4%), and 3/19 (15.8%), respectively. In HR-HPV group, it was found that that the frequency of sporadic lesions was 4/14 (28.6%), focal was 5/14 (35.7%), and diffuse was 5/14 (35.7%) (Table 3). There was a significant different between LR-HPV and HR-HPV groups in terms of p16^{INK4A} expression in CA lesion (p = 0,000). Correlation between p16^{INK4A} expression and LR-HPV and HR-HPV infections was moderate (r = 0,644, p = 0,000).

Discussion

The microscopic features of CA lesions based on haematoxylin eosin (HE) staining are parakeratosis, hyperkeratosis, hypergranulosis, basal cell hyperplasia, and koilocytic (Léonard et al., 2014). In this study, CA lesions were seen as papules, cauli flowers, and flat-tapping papules. The microscopic features found in this study were hyperkeratosis, parakeratosis, papillomatosis, hypergranulosis, and hyperplasia of basal cell, as well as the koilocytes that usually is associated

Table 1. Characteristics of Patients with Condyloma Acuminata

Characteristics of Patients	N (%)	Single or multiple infection LR/LR HPV N (%)	Multiple infection of LR/HR HPV N (%)
Sex			
Male	12 (36.4)	7 (21.2)	5 (15.1)
Female	21 (63.6)	12 (36.4)	9 (27.3)
Age			
15-24 years	17 (51.5)	10 (30.3)	7 (21.2)
25-34 years	6 (18.2)	3 (9.1)	3 (9.1)
35-44 years	7 (21.2)	4 (12.1)	3 (9.1)
45-54 years	2 (6.1)	1 (3.0)	1 (3.0)
55-64 years	1 (3.0)	1 (3.0)	0
Sexual Partner			
Heterosexuals	30 (90.9)	18 (54.5)	12 (36.4)
Homosexual	1 (3.0)	1 (3.0)	0
Bisexual	2 (6.1)	0	2 (6.1)
Duration of symptom			
< 1 month	5 (15.1)	1 (3.0)	4 (12.1)
1- 3 months	20 (61.6)	14 (42.4)	6 (18.2)
4 - 6 months	6 (18.2)	3 (9.1)	3 (9.1)
> 6 months	2 (6.1)	1 (3.0)	1 (3.0)
History			
First lesion	26 (78.8)	15 (45.4)	11 (33.3)
Recurrent lesion	7 (21.2)	4 (12.1)	3 (9.1)
Lesion on the partner			
Have lesion on the partner	4 (12.1)	2 (6.1)	2 (6.1)
No lesions on the partner	29 (87.9)	17 (51.5)	12 (36.4)
Therapy			
TCA	19 (57.6)	11 (33.3)	8 (24.2)
Cautery	14 (42.4)	8 (24.2)	6 (18.2)
Shape			
Papule	28 (84.8)	16 (48.5)	12 (36.4)
Cauli flower	4 (12.1)	2 (6.1)	2 (6.1)
Flat tapped papule	1 (3.0)	1 (3.0)	0
Type			
Multiple	31 (93.9)	18 (54.5)	13 (39.4)
Solitary	2 (6.1)	1 (3.0)	1 (3.0)
Location			
Penile	7 (21.2)	4 (12.1)	3 (9.1)
Anus	4 (12.1)	2 (6.1)	2 (6.1)
Penile and anus	1 (3.0)	1 (3.0)	0
Labia (majora and minora)	16 (48.5)	10	6 (18.2)
Vulva	3 (9.1)	1 (3.0)	2 (6.1)
Introitus vagina	1 (3.0)	0	1 (3.0)
Perineum	1 (3.0)	1 (3.0)	0

with HPV infection.

The most common cause of CA is infection by HPV. There are more than 40 genotypes of HPV that can infect the anogenital area that are usually infected by LR-HPV including HPV 6 and HPV 11 in single infection, but most commonly co-infection with LR-HPV or HR-HPV (Léonard et al., 2014; Hasanzadeh et al., 2019). The results of this study showed that all the tissues taken from the patients, who were clinically diagnosed with CA, were positive for LR-HPV or HR-HPV infections, in single infection or multiple infection. Most of the patients were infected by LR-HPV, while 42.4% were infected by

Table 2. Distribution of HPV in Condyloma Acuminata Lesions

Genotype of HPV	HR or LR HPV genotypes	Frequency N (%)
LR HPV		19 (57,6)
HPV 6	LR	4
HPV 11	LR	12
HPV 6, 11	LR	2
HPV 6,81,87,89	LR	1
HR HPV		14 (42,4)
HPV 6,11,18,51,82	HR	1
HPV 6,42,51,61	HR	1
HPV 11, 18	HR	2
HPV 11,18,45	HR	1
HPV 11, 26	HR	1
HPV 11,67	HR	1
HPV 11, 51, 82	HR	1
HPV 11, 52, 54	HR	1
HPV 11, 52,69,90	HR	1
HPV 11, 68B	HR	1
HPV 11, 82	HR	2
Total		33

LR-HPV and co-infected with HR-HPV. In line with this study, a cross-sectional study in Kuwait on 156 patients with genital warts showed that 102/156 (65.4%) of the patients were infected by LR-HPV and 54/156 (34.6%) of the patients were infected by HR-HPV. About 88.4% of the patients in the aforementioned study had single infection and 11.6% had multiple infections (Al-Awadhi et al., 2019). Another study in Spain showed that the frequency of LR-HPV infection was 63/138 (45.6%) in their patients and 71/138 (41.4%) of anogenital warts patients were infected by HR-HPV (Arroyo et al., 2016). The other study on 66 anogenital warts specimens showed that LR-HPV infected 42/66 (62.1%) which was dominated by HPV 6 (47%), and HPV-11 (13.6%), as well as HPV 18 and HPV 3 (Ozaydin-Yavuz et al., 2019). We discovered that that beside LR-HPV, CA was also co-infected with HR-HPV that could develop to malignant cancer. Therefore, identification of HPV genotype can predict the risk of developing related diseases. In addition, determination of HPV genotype affects treatment management, patients' follow-up, and prevention strategies

The findings of this study revealed that 57.6% of CA patients were infected by LR-HPV and the rest were infected by both LR-HPV and HR-HPV infections. The most common HPV genotypes were HPV 11 and HPV 6 for LR-HPV, followed by HPV 18, HPV 51, and HPV 82 for HR-HPV. The incidence of CA and the progression of the disease can be prevented by vaccination. Recently, there are 3 commercial vaccines against HPV infection, namely Gardasil to prevent infections caused by HPV 6, 11, 16, and 18, Cervarix to prevent infections caused by HPV 16, and 18, and Gardasil 9 to prevent infections caused by HPV 6, 11, 61, 18, 31, 33, 45, 52, and 58 (Gupta et al., 2017). A previous study on anogenital benign lesions showed that HPV genotype in CA patients was dominated by HPV 11 and HPV 6 among 13 female patients (Arista et al., 2019) and 12 male patients (Murtiastutik et al., 2019). In pre-cancerous lesion and cancerous lesion of uterine cervix, those were dominated by HPV 16 (62.68%), then followed by HPV 18 (20.9%), HPV 45 (5.97%), HPV 52 (5.97%), and HPV 67 (4.48%) (Mastutik et al., 2018). Vaccination programs is expected to reduce the incidence of these diseases, but there are some genotypes of HPV that cannot be targeted by the current vaccines. Therefore, strategies to prevent the incidence of CA or the progression of malignancy still need to be developed.

Oncoprotein E6 and E7 of HR-HPV play a role in cancer development. HR-HPV E6 mediates p53 inactivation by binding to the conserved domains of E6AP (E6-linked protein) to form the E6/ E6AP/p53 complex. This complex causes degradation of p53 by ubiquitination mechanism. HR-HPV E7 targets to degrade the retinoblastoma protein (pRB). In normal cells, when cells are prevented from entering the S phase, the pRB binds to the E2F family of transcription factor, so that the cell stops at the checkpoint of G1-S phase, activating cell cycles arrest. pRb, which is phosphorylated by cyclin D1/CDK 4/6 complex, causes E2F to be released and enter the nucleus. The cell enters the S phase which then starts the activation of gene transcription (Munger et al., 2013, Pal and Kudu, 2020). Furthermore, phosphorylated pRB is a $p16^{INK4A}$ feedback mechanism. HPV E7 induces degradation of pRB by an ubiquitin proteosome pathway that causes the loss feedback mechanism of $p16^{INK4A}$ and then leads to accumulation of $p16^{INK4A}$, presenting as overexpression of $p16^{INK4A}$ (Lassen et al., 2009; Faraji et al., 2017).

The overexpression of $p16^{INK4A}$ in this study was sporadic (39.4 %), focal (24.2%), diffuse (15.2%), and negative (21.2%). Another study on $p16^{INK4A}$ expression in 24 CA specimens showed that overexpression of

Table 3. The $p16^{INK4A}$ Expression on Condyloma Acuminata that Infected by LR or HR-HPV

$p16^{INK4A}$ expression	HPV genotype		Total	p-value
	LR-HPV N (%)	LR/HR-HPV N (%)	N (%)	
Score 0 (Negative)	7 (36.8%)	0 (0%)	7 (21.2%)	0.000*
Score 1 (Sporadic)	9 (47.4%)	4 (28.6%)	13 (39.4%)	
Score 2 (Focal)	3 (15.8%)	5 (35.7%)	8 (24.2%)	
Score 3 (Diffuse)	0 (0%)	5 (35.7%)	5 (15.2%)	
Total	19 (100%)	14 (100%)	33 (100%)	

*Mann Whitney, Asymptotic Significance (2-tailed) with p = 0,000 (p < 0,05); Correlation is significant at the 0.01 level (2-tailed) with Spearman's rho (r) = 0,644, p = 0,000)

p16^{INK4A} were sporadic in 11/24 of the specimens (45.8%), were focal in 7/24 of the specimens (29.2%), and were negative in 6/24 of the specimens (25%) (Kazlouskaya et al., 2013). The expression of p16^{INK4A} in cervix tended to increase from cervical normal epithelium to invasive cervical cancer (Missaoui et al., 2010; Izadi-Mood et al., 2012) and cervical adenocarcinoma (Mastutik et al., 2021). The expression of p16^{INK4A} in HR-HPV showed that all specimens were positive ranging from sporadic to diffuse. In LR-HPV were sporadic and focal, whereas 66.8 % of specimens were not expressed in p16^{INK4A} and none of specimens expressed in diffuse category. Other study on anal lesion with HR HPV infection showed that all specimens expressed p16^{INK4A} ranging from sporadic to diffuse, whereas those infected with LR-HPV were mostly patchy (Leeman et al., 2019). In oropharyngeal squamous cell carcinomas and tonsillar dysplasia that were positive for HPV 16, p16^{INK4A} expression was also diffuse. However, in benign and pre-malignant lesions were positive for HPV 6 and HPV 11 showed variations from negative to strong positive (Mooren et al., 2014). All cervical lesions infected with HR-HPV had a significant, strong, and diffuse p16^{INK4A} expression, whereas those infected with LR-HPV showed mild p16^{INK4A} expression (score 1) (Missaoui et al., 2014).

These findings highlighted that the expression of p16^{INK4A} significantly correlated with HR-HPV infection, in a way that CA lesions infected by HR-HPV had 0.644 times more chances to express p16^{INK4A} compared to CA lesions infected by LR-HPV. As previous studies showed that p16^{INK4A} expression was associated with HR-HPV infection in oropharyngeal squamous (Liu et al., 2015), mucosal squamous cell carcinomas of the head and neck (Antonsson et al., 2015), cervical squamous intraepithelial lesion (Yildiz et al., 2007), and invasive cervical and vaginal carcinomas (Missaoui et al., 2010; Missaoui et al., 2014). HR-HPV is integrated into the host cell genome, whereas LR-HPV prefers extra chromosome as episome, so that the expression of E6 and E7 oncoproteins are within the regulatory framework of E1 and E2 HPV (Boulet et al., 2007). HR-HPVs, such as HPV 18, 33, HPV 51, HPV 58, and HPV 59, were found to be integrated, while HPVs 30, 35, 39, 44, 45, 53, 56, 59, 74 and 82 did not tend to be integrated, but to be in episome form (Nkili-Meyong et al., 2019). In this study, it was found that HR-HPVs 18, 51, and 82. As reported by Nkili-Meyong et al., (2019), HPV 18 was founded to be integrated in 55% of the positive HPV 18 specimen, HPV 51 was integrated in 25% of the positive HPV 51 specimen, and HPV 82 was founded in extra chromosome. This integration was associated with partial or total deletion of E1 and E2 genes, leading to the overexpression of E6 and E7 due to loss of feedback mechanism by E2 protein (Woodman et al., 2007; Nkili-Meyong et al., 2019). In addition, the HR-HPV E7 oncoproteins have higher affinity to bind pRB than LR-HPV E7, increasing the accumulation of p16^{INK4A}. Therefore, all specimens infected by HR-HPV in this study were positive for the expression of p16^{INK4A} in sporadic, focal, and diffuse forms, while those infected by LR-HPV had sporadic and focal, but not diffuse expression.

In conclusion, this study found significant differences between CA specimens infected by LR-HPV (HPV 6, 11, 42, 61, 54, 81, 87, 89, 90) and HR-HPV (HPV 18, 26, 45, 51, 52, 67, 68B, 69, 82) regarding the expression of p16^{INK4A}. p16^{INK4A} expression was correlated with HR-HPV infection moderately. IHC is a routine method to perform the diagnostic of histopathological. Therefore, IHC of p16^{INK4A} could be used as biomarker for HR-HPV infection to predict the malignancy development of CA lesions.

Author Contribution Statement

Concepts, design, definition of intellectual content: Gondo Mastutik. Literature search: Gondo Mastutik, Afria Arista. Clinical studies: Gondo Mastutik, Dwi Murtiastutik, Trisniartami Setyaningrum. Experimental studies: Gondo Mastutik, Alphania Rahniayu, Afria Arista. Data acquisition: Nila Kurniasari, Alphania Rahniayu, Anny Setijo Rahaju. Data analysis: Gondo Mastutik, Alphania Rahniayu, Anny Setijo Rahaju.Statistical analysis: Gondo Mastutik, Nila Kurniasari, Anny Setijo Rahaju. Manuscript preparation and manuscript editing: Gondo Mastutik, Afria Arista, Anny Setijo Rahaju. Manuscript review: Gondo Mastutik, Alphania Rahniayu, Dwi Murtiastutik, Trisniartami Setyaningrum

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Ethical statement

Ethical study was approved by the Medical Ethic Research at Dr. Soetomo General Academic Hospital Surabaya, Indonesia (ethical code:382/Panke. KKE/V/2016).

Statement conflict of interest

The authors declare no conflict of interest

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