Author’s response to reviews

Title: Implication of Human Papilloma Virus-66 in vulvar carcinoma: a case report

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Author’s response to reviews:

Dear Prof. Michael Kidd,

Editor-in-Chief of the Journal of Medical Case Reports,

Thank you very much for the careful review of our manuscript entitled “MS: 3597680284076949- Implication of Human Papilloma Virus-66 in vulvar carcinoma: a case report” and your suggestion for resubmission after revision. We have revised our manuscript according to the reviewer’s suggestions and highlight these changes and other responses to Reviewer concerns, point by point, in bold, below.

We once again, would like to thank you for you patience and understanding regarding the delay of the revised manuscript. We are grateful for the opportunity to revise and resubmit and hope that our changes will be sufficient to secure publication in Journal of Medical Case Reports.

1. Reviewer: Dr. Reinhard R Kirnbauer

#1: Abstract: Introduction part: 1st line: „Vulvar carcinoma in elderly women is hardly associated with infection of any type of HPV“
The term „hardly may be replaced by „less often“.
Response: The term “hardly” has been replaced by “less often”

#2: 3rd line: „There is no reported case demonstrating and verifying direct and exclusive correlation between HPV-66 typing and vulvar cancer“.
The authors may want to cite this reference and change the above mentioned
sentence and adapt the introduction.

Response: The reference was cited and the introduction adapted accordingly, so that the paragraph now reads: “Reviewing the latest international literature, only one other case of HPV 66 and carcinoma coexistence is reported, in which however a combination of HPV 52 infection was present [1].”

#3: Case presentation part: 3rd line: „HPV66 genotype was detected through cytological examination and HPV DNA typing“. Detection of HPV 66 cannot be performed by cytological examination. Please correct.

Response: We have replaced this sentence with the following: “The presence of HPV infection was established by histological and cytological examination, followed by HPV-DNA typing, which revealed the presence of HPV 66 genotype.”

#4: Conclusion part: 3rd line. „Suspicious lesions should be treated likewise“. The authors may want to specify this statement.

Response: For the sake of brevity in the abstract we chose not to specify further; this has been restored as follows: “Suspicious lesions should be subjected to biopsy, and in the presence of carcinoma, vulvectomy by bilateral lymphadenectomy must be performed. PCR analysis with Clinical Arrays in cytological samples is an accurate test detecting a wide range of HPV genotypes and could be applied to verify the infection and specify the HPV type implicated”.

#5: Introduction: 1st line: „Vulvar carcinoma in elderly women is only rarely associated with any type of HPV infection. “…. The terms „only rarely“shall be omitted and be replaced by more precise data and a reference. The authors may want to define type 1 and 2 vulvar carcinoma.

Response: The term “only rarely” was replaced by the term “seldom”, and the sentence “representing less than 15% of reported cases” was added. According to data obtained from “Williams Gynecology” of Schorge J., Schaffer J., Halvorson L., Hoffman B., Brandshaw K. and Cunningham F.G., pages 665-668 (2nd reference in the manuscript), vulvar cancer is devised in type 1 and type 2 based on a number of characteristics, mainly on age but also on association to cervical neoplasia, cofactors, histology of tumor, HPV-DNA presence, pre-existing lesion etc. In particular, in table 31-2, type 2 vulvar cancer is mentioned to be mainly presented in older women (55-85 years) and there is seldom (<15%) presence of HPV-DNA.

The following have been added in the description of types 1 & 2: “Two models have been suggested in the development of vulvar cancer [1,2]. Type 1 occurs in relatively young women and is associated with warty or basaloid vulvar intraepithelial neoplasia. According to its definition, type 2 is represented by keratinizing squamous cell carcinoma”
#6: Case presentation: 1st line: „with a free obstetric and gynaecologic history” The authors need to specify this.

Response: Unfortunately, neither the patient nor her family was able to provide us with more accurate and detailed information about her clinical history. The sentence was rephrased as follows: “gravid 2 – para 2, with a free obstetric and gynaecologic history, is presented. After her second delivery, no data referring to the clinical history of the patient is available because the patient deterred from any preventive or diagnostic medical examination until now. Her last menstrual period was 28 years ago. Her medical history included hypertension and angina pectoris.”

#7: 7th line: „labia majus“. The singular is LABIUM MAJUS. Please correct. See also Figure legend 1

Response: We corrected this error from the manuscript.

#8: 3rd paragraph: „histopathological examination revealed the presence of SCC arising from a vulvar condyloma“. The authors may comment on invasion of the SCC. The terms “arising from a vulvar condyloma“shall be deleted and changed to VIN. Especially as Figure 3 shows “The adjacent squamous epithelium exhibited VIN-I and VIN-II”. The authors may want to correct this contradiction.

Response: More information about invasion was added: At the periphery of the lesion the squamous stratified epithelium exhibited abnormalities consistent with vulvar intraepithelial neoplasia (VIN I-II)” and “Carcinoma cells invaded the stroma and the underlying adipose tissue with irregular invasive margins (full thickness of the lesion, from the surface to the deepest point, 1.2 cm). As noted in the preoperative biopsies, the adjacent squamous epithelium exhibited VIN-I and VIN-II lesions (figure 3). Furthermore, metastases of the squamous cell carcinoma involving 2 out of 11 right inguinal lymph nodes and 2 out of 5 left inguinal lymph nodes were found.

#9: 4th para, 6th line: Please specify „focal to extended metastases“.

Response: Details about metastasis to inguinal lymph nodes were added: “Furthermore, metastases of the squamous cell carcinoma involving 2 out of 11 right inguinal lymph nodes and 2 out of 5 left inguinal lymph nodes were found.”

#10: 5th para „In the specimen obtained by liquid-based Cytology techniques…“ Where did the authors take the specimen for liquid-based cyto?

Can HPV 66 DNA also be detected e.g. by PCR and Clinical Arrays in the metastases of the regional lymph nodes? Additional experiments should have been performed to establish HPV as the causal link between tumor, metastases, VIN and condyloma. E.g. In situ hybridization experiments aimed at the detection of E6 and E7 transcripts and of the viral DNA in sections of the tumor
metastases, VIN and condyloma would have identified the cells expressing the viral genome and the cells productively infected (vegetative viral DNA replication).

Response: The sentence was rephrased: “In the specimen obtained preoperatively for the performance of liquid-based Cytology”. This technique was performed at the Cytology department of our Hospital. PCR was performed in the metastases of one of the affected lymph nodes, which was negative as expected.

#11: Conclusion: 1st Para, 1st line: „The relation of HPV-infection to anogenital squamous neoplasia in women is well established and usually encountered in patients with genital condylomatosis“. The latter statement is wrong and shall be deleted.

Response: The first sentence of the conclusion has been altered: “The presence, coexistence and possible causation of HPV-infection in women’s anogenital squamous neoplasia have been extensively studied over the last decade.”

#12: 2nd para, 1st line: „HPV-66 genotype is reported to be mainly associated with cervical squamous carcinoma“HPV 66 belongs to high-risk mucosal HPV types, but is also seen in benign lesions. See Ref: Virology. 2004 Jun 20;324(1):17-27. Classification of papillomaviruses. de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H.

Response: The intention under which this sentence was used was to emphasize the limited data concerning HPV66 infection and vulvar carcinoma, but we omitted to refer to its presence in benign lesions. The following alterations were made: “HPV-66 is an alpha-papilomavirus considered to belong among the potentially high-risk mucosal HPV types. Nevertheless, it can also be encountered in benign lesions. Even though this HPV type is reported to be associated with cervical squamous carcinoma, little is known concerning vulvar squamous cell carcinoma.”

#13: 3rd para „In conclusion, albeit rare, in patients with HPV-66 infection the possibility of a coexisting invasive malignancy, even in the presence of benign lesions, should be considered. “ My conclusion would rather be that Hybrid Capture II test does not detect all high-risk HPV types and that negativity does NOT rule out vulvar cancer, esp. in older women’s cancer, as the majority of these cancers are HPV negative. More sensitive tests like PCR with degenerate primers are required to establish HPV infections such as HPV 66 as causal.

Response: Taking under serious consideration your suggested conclusion as well as the additional information gathered with the PCR in tissue sections, we modified our conclusions as follows: “In conclusion, albeit rare, in patients with HPV-66 infection the possibility of a coexisting invasive malignancy, even in the presence of benign lesions, should be considered. Caution should be taken, especially in older women’s cancer, as the majority of these cancers are HPV
negative. PCR with Clinical Arrays should be considered in suspicious cases with histological features of HPV infection and negative Hybrid Capture II testing, as it detects a wider range of HPV genotypes including HPV 66. Standard PCR in formalin fixed samples seems to be less effective, as it appears to be affected by sampling, tissue fixation and/or viral load. Patients should be followed up meticulously at close time intervals.”

#14: Acknowledgements: “We recognize with respect Dr. Kartsiounis Christos, head of the department of gynecologic oncology for his help:"Please rewrite: „We recognize Dr. Kartsiounis Christos for his help” and specify his contribution.

Response: The phrase “with respect” was deleted, and Dr. Kartsiounis’ help was specified. Moreover, Mr. Kotsinas and Dr. Gorgoulis were added, as they performed the requested PCR, as well as Dr. Destouni, for her consultation on interpreting the cytological and PCR results.

15: Figures: The histological illustrations are good, however, Figure 1 seems to be a bit blurred. The authors may want to replace this picture.

Response: Unfortunately, this is the best figure we can provide. The quality of the others is not satisfactory.

#16: Figure legends. Figure 1. Labium majus

Response: The figure legend was corrected.

#17: Fig. 2.+3. Histological figures are poorly described. The authors may add something about invasion, dysplasia, inflammatory infiltrate etc...

Response: Details were added:
“Figure 2. Moderately-differentiated architectural and cytologic appearance of squamous cell carcinoma amongst mildly desmoplastic stroma (H&E x100)
Figure 3. Vulvar Intraepithelial Neoplasia (VIN-I) adjacent to carcinoma (H&E x100). There is proliferation and atypia of the lower third, but surface maturation is evident. The stroma is heavily infiltrated.”

#18: Figure 4. „Koilocytotic changes in vulvar squamous epithelium. These are diagnostic of HPV infection"Replace “diagnostic” with “consistent with”

Response: The word “diagnostic” was replaced by the phrase “consistent with”.

#19: Figure 5. „Clinical Arrays Technique used for HPV typing. The combination of the three dark diagonal points indicated the presence of the HPV-66 typing“The authors should explain all the other less prominent dots.
Response: Further explanations were added (they represent markers control).

2. Reviewer: Dr. Jorge Fabricio Gonzalez

#1: The authors do not have confirmed their results with other molecular tests, commercials and non-commercials, to contrast their results, specific to HPV66. How is usual in a molecular genetics laboratory the results may vary between some methods used, especially in the diagnosis of viruses (genetic drift and genetic shift). It recommends confirm a unique or unusual result with at least two different methods of analysis (RFLPs, standard PCR, sequencing, arrays, real-time PCR, etc).

Response: As requested, we performed standard PCR in multiple specimens. Four specimens at different level of the main lesion, and one specimen from a lymph node with metastasis were tested. The reason why Hybrid Capture 2 didn’t identify HPV66 was technical as, this method could not identify this serotype. On the other hand, even though the performed PCR was negative in all specimens, detection of the IFN-# gene product amplification is consistent with the presence of HPV infection with possible oncogenic potential (Song SH, Lee JK, Seok OS, Saw HS. The relationship between cytokines and HPV-16, HPV-16 E6, E7, and high-risk HPV viral load in the uterine cervix. Gynecol Oncol. 2007 Mar;104(3):732-8. Epub 2006 Dec 22).

#2: In the abstract, the authors say HPV66 genotype was detected through cytological examination? I guess there is a mistake in this affirmation.

Response: please see reply to reviewer 1, comment 3.

#3: They have to consider that it exist the probability of co-existence of 2 or 3 HPV genotypes in the same lesion. How the authors discard several HPV genotypes in the same sample? HPV 66 was associated with atypical cytology and was found in women with borderline cytology, low-grade lesions and high-grade lesions, but was most frequent in the threshold group. One frequent mistake is to analyze similar samples of the same macroscopic piece. It recommends take different samples, at different levels of the lesion and, at different location (lesion and surrounding areas), if the authors try to demonstrate the correlation of one specific HPV genotype in a pathological lesion. In that case and if the authors did this procedure, it is necessary a detailed description of the sampling and the genotyping of each one.

Response: Sampling included different levels and sites of the lesion as now explained in the manuscript. Neither PCR with Clinical Arrays and Hybrid Capture 2 HPV-DNA test, nor standard PCR of tissue samples detected any
other type of HPV. We excluded the probability of co-existence of more HPV genotypes as they would have been identified with at least one of these methods.

#4: It has described more than 200 different HPV-genotypes associated with genital lesions. How the authors discard all others genotypes, if the array only analyzed 35 or 40 genotypes? In 2009, IARC downgraded HPV66 from the category of probably carcinogenic (Group 2A), as judged in 2005, to possibly carcinogenic (Group 2B), which also includes HPV genotypes HPV26, HPV53, HPV67, HPV70, HPV73, and HPV82

Response: PCR with Clinical Arrays has the ability to detect accurately a wide range of genotypes that investigators probably most commonly come across. Unfortunately aside from the use of standard PCR and especially in the every day practice, we do not have the means to detect all known types of HPV.

#5: The paper is poorly presented. It is necessary a more specific introduction, with at least 20 current references. It will be necessary a detailed description of the genetic methods used in the analysis (genotypes analyzed, sensibility and specificity, limitations of that laboratory analysis, etc.). It is absolutely necessary to write a small discussion about this unique finding, with strong arguments that defends this particular etiology and correlation.

Response: Even though we would have liked to use more references in the introduction as well as in the rest of the manuscript, according to the “Instructions for JMCR authors”, references should be no more than 15. So we tried to keep the number of the references under this limit. As you wisely suggested we described the used genetic methods with more details (please see manuscript : case presentation, 6th paragraph).

#6: In the case presentation, it’s necessary to explain the past clinical history of the patients, searching other risk factors and, it will be interesting to get previous Pap-test results (of 5 or 10 years ago), to try to identify the chronology of this pathology.

Response: please see reply to reviewer 1, comment 6.