論 文 要 旨

THE PREVALENCE OF THE HUMAN PAPILLOMAVIRUS AND ITS COFACTORS IN BREAST CARCINOMAS TO EVALUATE ITS ROLE IN BREAST CARCINOGENESIS

（乳がんにおけるヒトパピローマウイルスの分布および発がん過程におけるその役割と関連要因に関する検討）

Noureen Afsar Khan

ABSTRACT

Purpose of this study

Recent studies have revealed a possible association with human papillomavirus (HPV) in the pathogenesis of breast cancer. To investigate the etiological role of HPV in breast cancer, we examined the presence, genotype, viral load, and physical status of HPV in breast carcinomas (BCs) collected from different countries.

Subjects and methods

The present study examined 307 BCs collected from Japan (n=124), Pakistan (n=61), India (n=11), Mexico (n=63), and Chile (n=48). Cervical carcinoma (CC) samples from Japan and Pakistan were also used for comparison. HPV presence was examined by PCR using SPF10 primers, and primer sets targeting the *E6* region of HPV-16, -18, and -33. The INNO-LiPA HPV genotyping kit was used to determine genotype. Real-time PCR analysis was used to examine viral load and physical status of HPV DNA. The $p16^{INK4a}$ and p53 expressions were analyzed by immunohistochemistry assays.

Results

1. HPV detection rate

HPV DNA was detected in 26 (21%), 17 (28%), 4 (36%), 8 (13%), and 5 (10%) BCs from Japan, Pakistan, India, Mexico and Chile, respectively. The difference of HPV detection rate among these countries was statistically significant (P=0.048). In CCs, HPV was detected in 72 (85%) Japanese and 77 (94%) Pakistani cases. The presence of HPV was significantly different between the breast and CC ($P < 0.001$). The most frequently detected HPV genotype was HPV-16 in both sites, and its frequency was 54 (90%) and 125 (84%) carcinomas of breast and cervix, respectively. The detection rate of HPV-16 in all HPV-positive cases was not different between breast and CC.

2. Distribution of HPV genotypes

In all HPV-positive cases with BCs, the frequency of low-risk HPV genotype (P=0.021) and HPV multiple infection (P=0.032) was significantly different among the five countries. Most cases with low-risk HPV infection were revealed to harbor multiple HPV types (93%), and the highest frequencies of low-risk types and multiple infections of HPV were observed in Japanese series (46% for both). Multiple infections were also found in 2 (12%) and 1 (13%) BCs of Pakistan and Mexico, respectively. In CC, multiple infection was more frequently found when compared to BCs ($P < 0.001$): 91% in Pakistan and 15% in Japan. The observed difference between the two countries was statistically significant ($P < 0.001$).

3. HPV detection in normal tissue and breast milk

In 19 normal epithelium specimens adjacent to 19 HPV-16-positive BCs, 10 (53%) were HPV-16-positive. However, three (5%) of the normal breast tissue specimens adjacent to HPV-negative BCs were also HPV-positive. Nipple specimens adjacent to eight HPV-16-positive Mexican BCs were also examined, and one nipple specimen was HPV-16-positive. In addition, ten clostrum and 25 breast milk samples from Japan were also examined for HPV presence. One (10%) clostrum specimen was positive for HPV-16 and two (8%) breast milk samples were positive for HPV. However, only low-risk types of HPV were detected in milk samples.

4. Viral load and physical status

To clarify the etiological involvement of HPV in carcinogenesis of the breast, further analyses were conducted. Real-time PCR analysis suggested the presence of integrated-form of viral DNA in all HPV-16-positive BCs from all countries, and estimated viral load was low with geometric mean of 5.4, 1595, 158, 5.3 and 6402 copies per 10^4 cells in carcinomas of Japan, Pakistan, India, Mexico and Chile, respectively. The geometric mean of HPV-16 viral load was significantly different among these countries ($P < 0.001$). All HPV-16 positive CCs were found integrated into the host genome except two cases, which were suspected to have only episomal HPV-16. The geometric mean of HPV-16 viral load in cervical cancers of Japan and Pakistan was 410,000 and 2,370,000 copies per 10^4 cells, respectively. The geometric mean of viral load in the CC was higher than BC and the difference was statistically significant.

5. Immunostainning

The $p16^{INK4a}$ expression was significantly high in HPV-positive CCs ($P = 0.031$). Although a similar association was found in BC, their relationship was not statistically significant. On the other hand, p53 expression tended to decrease in HPV-positive CCs ($P = 0.110$), while there was no trend in BC. These differences of $p16^{INK4a}$ and p53 expressions between cervical and BCs were statistically significant ($P = 0.013$ and $P < 0.001$, respectively).

Conclusion

In conclusion, the relatively low HPV copy number and infection rate in breast cancer suggest that HPV is unlikely to play as an essential role in the carcinogenesis of breast cancer as in genital neoplasia. However, since oncogenic HPVs were consistently detected in BC and most of the detected HPV-16 DNA was considered integrated into the host genome, HPV infection may play some roles in the carcinogenesis of a subset of BCs.

最終試験の結果の要旨

報告番号	総研第 71 号		学位申請者	Noureen Afsar Khan
審査委員	主査	竹内 亨	学位	博士（(医学)・歯学・学術）
	副査	小田 紘	副査	金蔵 拓郎
	副査	秋山 伸一	副査	米澤 傑

主査および副査の5名は、平成21年8月4日、学位申請者 Noureen Afsar Khan 君に面接し、学位申請論文の内容について説明を求めると共に、関連事項について試問を行った。具体的には、以下のような質疑応答がなされ、いずれについても満足すべき回答を得ることができた。

Q1. Human papillomavirus (HPV)-positive breast carcinoma (BC) seems to be more frequent in European people than Asian people. Is this related to the high incidence of BC in Europe?

A1. There are five studies from Europe on the prevalence of HPV in BCs. The prevalence in Europe varies from 0 to 74%. Furthermore, in two independent studies, HPV16 was found to be present with 47% of BC European patients who have had HPV16-associated cervical cancer before. High prevalence of HPV may be related to high frequency of BC in Europe but we need the strong evidence based on prospective studies.

Q2. Japanese immigrants to Hawaii has higher BC incidence. Is it related to HPV infection? Are there any data of HPV prevalence among the Japanese immigrants, either in BC or in any other cancers?

A2. Since there is no study in the literature reporting HPV prevalence in BC and other cancers among Japanese immigrants, we cannot relate the higher BC incidence in Japanese immigrants to Hawaii with HPV.

Q3. How does HPV infect to breast epithelial cells, in the same manner at cervix?

A3. As great majority of primary tumors have proved difficult to establish in cultures, there is no evidential study showing that HPV infection occurs in the same manner between cervical and breast epithelial cells. Unlike cervical epithelial cells, HPV genotypes detected in BCs probably originate from luminal, basal, and possibly, stem cell compartment. Since breast stem cells have beta-6 integrin, one of the candidates of HPV receptor, HPV may infect the progenitor stem cells and the secretary cells.

Q4. What is the HPV transmission route in BC?

A4. The transmission route of HPV in BC is yet unclear. But two independent studies suggested possible hematogenic and /or lymphatic transfer from one organ to another. On the other hand, de Villiers *et al.* (2005) showed HPV presence in the nipple, suggesting HPV transfer in a retrograde fashion from the nipple via areola, lactiferous ducts, and sinuses.

Q5. Are there any studies of vertical transmission of HPV via breast milk?

A5. There is no study showing the HPV transmission from mother to infant by milk. The reported studies showed only the presence of HPV in the breast milk.

Q6. What is the major histological type of BC in your study? Do you find any difference in HPV detection, genotype, viral load, and physical status among different histological types?

A6. We studied mainly adenocarcinoma. Thus, it is hard to compare viral factors with pathological types of BC.

Q7. What do you mean multiple infections? Are there any significant difference in HPV genotype or viral load between multiple and single infection of HPV?

A7. Multiple infection means coinfection of more than one HPV genotype in cancer tissues. Becker *et al.* (1994) found that multiple HPV infections increase the risk of cervical dysplasia. In addition, multiple HPV infections may reflect loss of immune response of the host as well as persistent HPV infection.

論文審査の要旨

報告番号	総研第 71 号		学位申請者	Noureen Afsar Khan
審査委員	主査	竹内 亨	学位	博士（医学・歯学・学術）
	副査	小田 紘	副査	金蔵 拓郎
	副査	秋山 伸一	副査	米澤 傑

The prevalence of the human papillomavirus and its cofactors in breast carcinomas to evaluate its role in breast carcinogenesis

乳がんにおけるヒトパピローマウイルスの分布およびウイルス側の要因と発がん過程におけるその役割に関する検討

高リスク型のヒトパピローマウイルス（HPV）が子宮頸がんの原因ウイルスとしての役割を果たしていることが明らかとなり、他の臓器のがんにおける HPV の役割も注目されている。近年、乳がん組織からも HPV ゲノムが検出されるという報告もあるが、HPV の病因学的役割については不明である。そこで学位申請者は、乳腺の悪性新生物における HPV の病因学的役割を明らかにすることを目的として、5 つの国（日本、パキスタン、インド、メキシコ、チリ）から得られた乳がんの病理標本を用い、HPV ゲノムの検出とそのサブタイプや variant の分布、ウイルス量と宿主ゲノムへの組み込みの有無を解析した。さらに一部の HPV 陽性例と陰性例において p16 や p53 発現頻度の違いを検討した。

対象は、日本 124 例、パキスタン 61 例、インド 11 例、メキシコ 63 例、チリ 48 例（計 307 例）の乳がんである。陽性コントロールとして子宮頸がんの病理標本を、日本の症例ではがん周囲の正常乳腺組織の病理標本や母乳（非がん女性）も解析に用いている。HPV ゲノムの検出は SPF10 プライマーを用い、INNO-LiPA HPV genotyping キットにより HPV の型を判定した。さらに、HPV16、HPV18、HPV33 の E6 領域を標的としたプライマーによる検出も行っている。HPV16 陽性例については、real-time PCR 法にてウイルスを定量し、HPV E2 と E6 のコピー数の比を求めることにより宿主ゲノムへの組み込みの有無を確認した。また一部の症例については、免疫組織化学染色法にて p16 と p53 の蛋白発現頻度を調べた。その結果、本研究で以下の知見が明らかとなった。

1） 乳がん組織中の HPV ゲノム検出率は 10－36％と地域差があるものの、いずれの地域においても HPV 陽性例のほとんどが高リスク型の HPV16 であった。
2） 低リスク型の HPV ゲノムも検出されたが、そのほとんどは高リスク型 HPV との重複感染であった。また、重複感染の頻度は日本の症例で 46％と最も多く、地域差が認められた。
3） HPV16 陽性である 11 例中 7 例の正常組織中にも HPV16 ゲノムが確認された。また、初乳と母乳を調べた結果、いずれも約 1 割の頻度で低リスク型 HPV ゲノムが検出された。
4） 乳がん組織中の HPV コピー数は細胞 10^4 個あたりの幾何平均は 5－6000 コピーと子宮頸がん（41 万－240 万コピー）よりもはるかに少なく、地域間のばらつきも大きかった。一方、いずれの HPV16 陽性例においても宿主ゲノムへの組み込みが確認された。
5） HPV 陽性乳がんでは、子宮頸がん同様、p16 の発現頻度が高い傾向が認められたが、統計学的有意差はなかった。一方、p53 の発現頻度は HPV 陰性乳がんと同様であった。

本研究は、乳がん組織中の HPV のコピー数と宿主ゲノムへの組み込みを初めて確認したものであり、乳腺の発がん過程における HPV の関与を検討する上で重要な知見を与えるものと考える。乳がん組織中の HPV コピー数は非常に少ないものの、すべての HPV16 陽性例において宿主ゲノムへの組み込みが認められることや、いずれの地域においても一定の頻度で高リスク型 HPV が検出されることは、発がん過程において HPV が何らかの役割を果たしている可能性を示唆している。

よって本研究は学位論文として十分な価値を有するものと判定した。

最終試験の結果の要旨

Q8.What are HPV cofactors?
A8. HPV cofactors are genotypes, multiple/single infections, variants, viral load, and physical status of HPV.

Q9. Is episomal form of HPV stable?
A9. During the HPV integration, the opening of episomal viral ring molecule takes place and showed the unstable nature of episomal form. Pett and Coleman (2007) proposed a model that HPV episomal genome is eliminated by the host innate immunity, leaving HPV-integrated host cells.

Q10. How does HPV integrate into host genome without E2 disruption?
A10. Although E2 disruption mainly occurs, sometimes E1 or L1 disruption takes place. Anyhow, the part of HPV genome got disrupted during integration.

Q11. Why didn't you observe a relationship between HPV presence and HER2/Neu overexpression, which contradicts the result reported by Yasmeen *et al*?
A11. We checked the HER2 expression in clinical specimens by immunohistochemistry. In contrast, Yasmeen *et al.* used ErbB-2/E6/E7 double transgenic mice and checked the interactive effect of ErbB-2 and high-risk HPV infection in breast carcinogenesis. Because of different study subjects, clinical specimens with low viral load versus transgenic mice, we could not confirm their results.

Q12. Are there any associations between hormone receptors and HPV genotype?
A12. In our study, almost all the HPV-positive BC cases were HPV16-positive, and we did not find any association between hormone receptors and HPV genotype.

Q13. Does HPV have hormone response elements?
A13. There are hormone response elements in the long control region of the virus. Steroid hormone can interact with the elements and enhance HPV transcription.

Q14. Are there any relationships between the HPV infection and BC progression?
A14. Since HPV16 E6/E7 proteins bind to the promoter of Id-1, which regulates cell invasion and metastasis of human breast cancer cells (Desprez et al., 2004; Fong et al., 2003), the virus may play important roles in the regulation of Id-1and induction of cell invasion and metastasis. However, we did not examine Id-1 in this study. Furthermore, Yasmeen *et al.* reported a significant association of HPV16 and invasive BC, and showed HPV16 E6/E7 expressing MCF7 and BT20 cells increased cell invasiveness and metastasis in vitro and in vivo, respectively, in comparison with wild type of cells.

Q15. How do you interpret the low viral load in BCs?
A15. Our results showed a frequent integration of HPV16 in the host genome, indicating HPV infection before cancer development since new HPV infections are characterized to have an episomal form. Low viral load may be the result of hit and run mechanism.

Q16. Koilocytosis is observed in cervical cancers but not in BCs. How do you explain the difference?
A16. HPV E6/E7, which act early in transformation, are known to disrupt cytokeratin causing perinuclear cytoplasmic clearing and nuclear enlargement which leads to the appearance of a koilocytes. We could not find koilocytosis in Japanese BCs whose viral loads were very low, 5.4 copies/10^4 cells. Low E6 and E7 expressions may not be enough to induce koilocytes in BCs.

Q17. SPF10 primer sets targeting L1 gene, which may be lost during integration. Are there better target regions rather than L1?
A17. HPV E6 and E7 genes are best targets along with the consensus primers, and I also used primers targeting E6.

Q18. When you limited the literature using similar detection methods with yours, do you see any difference in the HPV detection rate in BCs?
A18. There are five studies using similar method to ours, and their HPV prevalence ranged from 16 to 74%. We found HPV in 20% of the BCs from five different countries and our result is in the reported range.

以上の結果から、5 名の審査委員は申請者が大学院博士課程修了者としての学力・識見を有しているものと認め、博士（医学）の学位を与えるに足る資格を有するものと認定した。