Table 3
MICs of fluconazole, oxiconazole, isoconazole, clotrimazole, and miconazole for *C. albicans*, *C. parapsilosis* and *C. glabrata* isolates.

	C. albicans (N = 100)			*C. parapsilosis* (N = 2)			*C. glabrata* (N = 1)		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
Fluconazole	0.12–4	0.25	0.25	1–1	–	–	8–8	–	–
Oxiconazole	0.06–0.25	0.06	0.06	0.25–0.5	–	–	0.06–0.06	–	–
Isoconazole	0.06–0.25	0.06	0.06	0.5–0.5	–	–	0.5–0.5	–	–
Clotrimazole	0.06–0.06	0.06	0.06	0.06–0.06	–	–	0.5–0.5	–	–
Miconazole	0.06–1	0.06	0.06	0.5–0.5	–	–	0.5–0.5	–	–

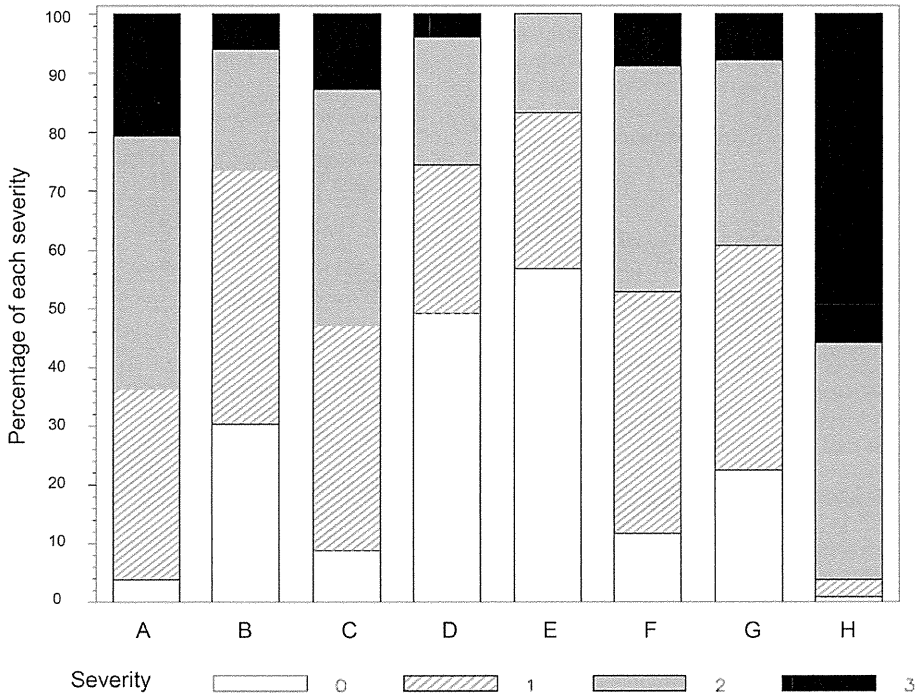

Fig. 1. Percentage graphs of severity scores of clinical signs and symptoms at baseline in the m-ITT population. Clinical signs and symptoms: A = vulvovaginal itching, B = vulvovaginal burning sensation, C = vaginal discharge, D = excoriation, E = vulval edema, F = redness of vulva, G = vaginal redness, and H = Property of vaginal content. Scores: 0 = normal, 1 = mild, 2 = moderate, and 3 = severe in A to G; and 0 = normal, 1 = mucoid, 2 = paste-like, and 3 = cottage cheese-like, cheese-like or granular in H.

The most common treatment-related adverse events were diarrhea and nausea (1.9% each). All adverse events were mild or moderate in severity, and no deaths, serious adverse events, or discontinuations due to treatment-related adverse event were reported.

4. Discussion

Overall, in this non-comparative phase 3 study, a single oral 150 mg dose of fluconazole was effective for the treatment of Japanese subjects with vulvovaginal candidiasis.

The efficacy rate for the therapeutic outcome on Day 28 in the m-ITT population in this study was comparable to the clinical efficacy rates of a single oral 150 mg dose of fluconazole (80% at short-term assessment [Days 5–15] and 76% at long-term assessment [Days 30–60]) reported in previous clinical trials in Japanese subjects with vulvovaginal candidiasis [9,10]. The mycological efficacy on Day 28 in the present study was higher than 76% (short-term assessment) and 70% (long-term assessment) in previous Japanese studies. The difference in mycological efficacy between the current and previous studies may partly be due to the difference in the assessment time (Day 28 versus Days 5–15 or 30–60).

Table 4
Therapeutic outcome in the m-ITT population.

Assessment time point	N	Therapeutic outcome				
		Effective n (%)	Ineffective n (%)	Indeterminate n (%)	Efficacy rate[a] (%)	95% CI (%)
Day 7	95	31 (32.6)	61 (64.2)	3 (3.2)	33.7	24.2–44.3
Day 14	100	52 (52.0)	44 (44.0)	4 (4.0)	54.2	43.7–64.4
Day 28	102	74 (72.5)	25 (24.5)	3 (2.9)	74.7	65.0–82.9

m-ITT, modified intent-to-treat; CI, confidence interval.
[a] Efficacy rate (%) = (number of subjects considered effective/[number of evaluated subjects − number of subjects considered indeterminate]) × 100.

Table 5
Clinical efficacy in the m-ITT population.

Assessment time point	N	Clinical efficacy							
		Cure n (%)	Improvement n (%)	Ineffective n (%)	Indeterminate n (%)	Cure rate[a]		Cure or improvement rate[b]	
						%	95% CI (%)	%	95% CI (%)
Day 7	99	32 (32.3)	60 (60.6)	0	7 (7.1)	34.8	25.1–45.4	100.0	96.1–100.0
Day 14	101	55 (54.5)	40 (39.6)	1 (1.0)	5 (5.0)	57.3	46.8–67.3	99.0	94.3–100.0
Day 28	102	80 (78.4)	14 (13.7)	4 (3.9)	4 (3.9)	81.6	72.5–88.7	95.9	89.9–98.9

m-ITT, modified intent-to-treat; CI = confidence interval.
[a] Cure rate (%) = (number of subjects considered cure/[number of evaluated subjects − number of subjects considered indeterminate]) × 100.
[b] Cure or improvement rate (%) = (number of subjects considered cure or improvement/[number of evaluated subjects − number of subjects considered indeterminate]) × 100.

Table 6
Mycological efficacy in the m-ITT population.

Assessment time point	N	Mycological efficacy				
		Eradication n (%)	Persistence n (%)	Indeterminate n (%)	Eradication rate[a]	
					(%)	95% CI (%)
Day 7	95	90 (94.7)	4 (4.2)	1 (1.1)	95.7	89.5–98.8
Day 14	100	88 (88.0)	10 (10.0)	2 (2.0)	89.8	82.0–95.0
Day 28	102	85 (83.3)	14 (13.7)	3 (2.9)	85.9	77.4–92.0

m-ITT, modified intent-to-treat; CI = confidence interval.
[a] Eradication rate (%) = (number of subjects considered eradication/[number of evaluated subjects − number of subjects considered indeterminate]) × 100.

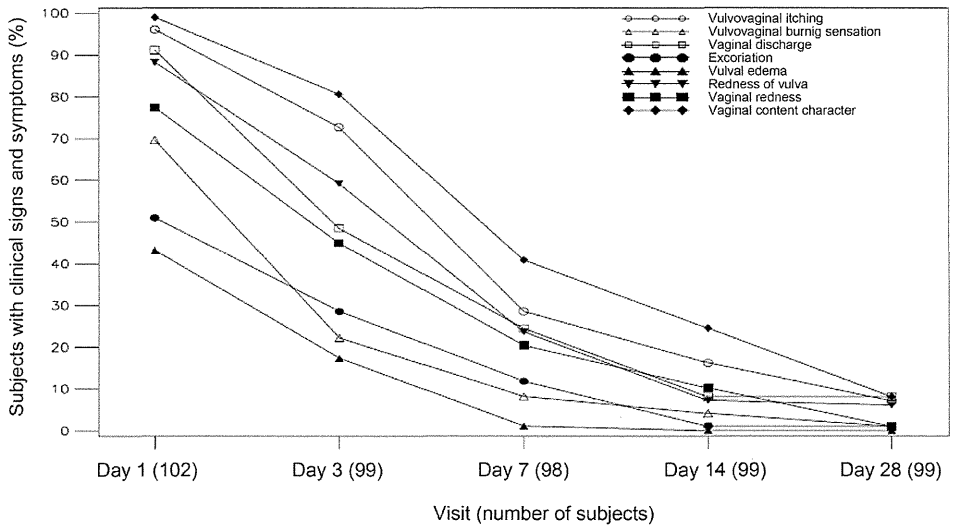

Fig. 2. Changes in the percentages of subjects with clinical signs and symptoms. Percentage = (number of subjects with clinical signs and symptoms/[N − NA]) × 100. NA, number of subjects whose data were not available due to menstruation.

Similarly, in 2 global clinical studies of a single oral 150 mg dose of fluconazole, the clinical efficacy rates (97 and 94%, respectively) at the short-term follow-up visit (Day 7 and Days 5–16, respectively) remained at a high level (87 and 84%, respectively) at the long-term follow-up visit (Day 21 and Days 26–50, respectively), while the mycological efficacy rates were lower at the long-term follow-up visit (76 and 59%, respectively) than those at the short-term follow-up visit (97 and 81%, respectively) [11,12]. This may reflect that the indigenous flora of the vagina that was affected once by a single oral dose of fluconazole returned to normal after administration [5]. In other words, mycological assessment at the long-term follow-up visit detected *Candida* as normal vaginal flora.

Mizuno et al. conducted 2 clinical studies of vaginal tablets [13,14]. They reported the cure rates and improvement rates at 3 weeks after the treatment were respectively 61.5% and 92.3% for 1-day treatment with oxiconazole, 72.8% and 95.7% for 6-day treatment with oxiconazole, 63.3% and 90.8% for 1-day treatment with isoconazole, and 73.9% and 95.7% for 6-day treatment with clotrimazole. Mikamo et al. also reported the clinical efficacy of clotrimazole was 58% on Days 30–60 [9,10]. Although the evaluation methods and timing in these studies were different from those in our study, the results of our study were generally consistent with those of these studies.

A single oral 150 mg dose of fluconazole is an easy-to-manage dosing regimen and has many advantages. Research based on interviews with prescription doctors in the UK revealed that 27% of patients complaint of pain and difficulties with insertion of vaginal tablets and pessary, and 62% of prescription doctors also think there

Table 7
Treatment-related adverse events in the safety analysis set.

Preferred term[a]	Safety analysis set (N = 157)
	Treatment-related adverse event n (%)
Palpitations	1 (0.6)
Abdominal distension	1 (0.6)
Diarrhea	3 (1.9)
Nausea	3 (1.9)
Pyrexia	1 (0.6)
Cystitis	1 (0.6)
Genital herpes	1 (0.6)
Hepatic enzyme increased	1 (0.6)
Genital hemorrhage	1 (0.6)
Urticaria	1 (0.6)

[a] Medical Dictionary for Regulatory Activities Terminology version 16.1.

is a problem with leakage of vaginal cream [6]. There are also some reports stating easy-to-administer oral therapies are preferred to topical vaginal therapies among patients [5,7]. In this study, vulvovaginal redness, irritation, tingling, itching, and pain were not reported as adverse events, suggesting that a single oral 150 mg dose of fluconazole had little adverse effect on the vulva and vagina and the daily life of subjects compared with topical therapies, and thus provides the improved quality of life and satisfaction of patients besides resolving the difficulties with topical drug administration.

Symptoms of vulvovaginal candidiasis often develop before menstruation [15]. It is difficult to apply topical vaginal therapies before and during menstruation, when a sufficient effect of treatment cannot be expected because of leakage and reduction in drug contents caused by menstrual bleeding. Single oral administration of fluconazole can be used regardless of the menstrual status of patients.

It is known that 6-day topical vaginal therapies show problems of early discontinuation of treatment for the reason of disappearance of symptoms and accidental dislodgement of inserted vaginal tablets that are not in place, leading to reduce compliance. With a single oral dose of fluconazole, there is no concern about such problems.

A single oral 150 mg dose of fluconazole can be prescribed at clinics of internal medicine as well as obstetrics and gynecology, raising concern that some clinicians may make a diagnosis of vulvovaginal candidiasis based on the patient's complaints without confirming the vulvovaginal findings, and prescribe the drug, resulting in delayed proper treatment of similar diseases such as vaginal trichomoniasis and bacterial vaginosis. To prevent inappropriate use, clinicians should make a definite diagnosis of the disease giving the utmost care before prescribing the drug.

In this study, 4 subjects had complications of genital infection caused by other pathogens than *Candida* (1 with *Trichomonas* vulvovaginitis, 3 with chlamydial cervicitis) at baseline. These complications were found in the microbiological tests conducted at baseline after a single oral 150 mg dose of fluconazole on Day 1. A subject with trichomonas vulvovaginitis received tinidazole and 3 subjects with chlamydial cervicitis received azithromycin. The investigator considered that it was possible to assess the efficacy of fluconazole in these 4 subjects. For these 4 subjects, the clinical efficacy was assessed as either "cure" or "improvement," and the mycological efficacy was assessed as either "eradication" or "persistence." For 3 of the 4 subjects, the therapeutic outcomes were assessed as "ineffective." These findings suggest that a single oral 150 mg dose of fluconazole in combination with other antimicrobial agents is effective for the treatment of vulvovaginal candidiasis complicated with genital infection caused by other pathogens than *Candida*.

A subject had complications of diabetes mellitus in this study. Although the subject was considered to have had them before enrollment, the investigator considered that it was possible to assess the efficacy of fluconazole in the subject because the duration of diabetes mellitus was short, and the subject was considered not to be immunocompromised. The therapeutic outcome of the subject was assessed as "effective." There were few studies reporting the efficacy of a single oral dose of fluconazole in subjects complicated with diabetes mellitus [16,17]. As diabetes mellitus is a risk factor for vulvovaginal candidiasis, the efficacy in these subjects has to be studied further.

In this study, the most commonly identified pathogen was *C. albicans* (100/104 strains), and all isolates were highly susceptible to fluconazole judging from the fluconazole breakpoints for *C. albicans* in the criteria of Clinical and Laboratory Standards Institute (CLSI) (susceptible $\leq$ 2 μg/ml, susceptible-dose dependent = 4 μg/ml, resistant $\geq$ 8 μg/ml) [18], and the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (susceptible $\leq$ 2 μg/ml, resistant $\geq$ 4 μg/ml) [19]. In this study, *C. glabrata* was identified in a subject whose therapeutic outcome was assessed as "effective" at all assessment time points. This *C. glabrata* isolate (MIC of fluconazole was 8 μg/ml) was not classified into the category of resistant based on the fluconazole breakpoints for *C. glabrata* (susceptible-dose dependent $\leq$32 μg/ml, resistant $\geq$ 64 μg/ml [CLSI criteria]; susceptible $\leq$ 0.002 μg/ml, resistant > 32 μg/ml [EUCAST criteria]). *C. glabrata*, the second most common pathogen of vulvovaginal candidiasis following *C. albicans*, is however reported to be resistant to fluconazole [20,21]. If fluconazole is not effective, other topical drugs should be used instead.

The most common treatment-related adverse events occurring in 1% or more of the subjects were only gastrointestinal-related events (diarrhea and nausea), and there were no significant safety issues in this study. However, fluconazole, even given as a single oral dose, has more potential to produce side effects such as gastrointestinal disorders and other systemic events compared with topical vaginal therapies, and subjects treated with fluconazole should be monitored carefully. Since fluconazole inhibits CYP 2C9 and 3A4, caution should be exercised when a single oral dose of fluconazole is applied in combination with other drugs to prevent potential drug interactions.

There are 3 limitations to this study that should be considered when these findings are interpreted. First, this was not a controlled study. Second, there were not sufficient other pathogens than *C. albicans* to assess their efficacy. Although further investigation, such as a large comparative study, accordingly needs to be performed to confirm our study results, the methods of this study with sufficient clinical efficacy will be useful for the treatment of patients with vulvovaginal candidiasis in Japan. Third, this was a clinical study led by the sponsor and the possibility of conflicts of interest between the authors and the sponsor could not be ruled out.

In conclusion, the results of this study demonstrated that a single oral 150 mg dose of fluconazole was effective for the treatment of vulvovaginal candidiasis in Japanese subjects, and no significant safety issues were reported. A single oral 150 mg dose of fluconazole is recommended for the treatment of vulvovaginal candidiasis in global guidelines. Therefore, a single oral 150 mg dose of fluconazole will be positioned as one of the first-line treatment options for vulvovaginal candidiasis in Japan.

Conflict of interest

H. Mikamo has received a consultant fee and a fee for participation in the Committee from Pfizer Japan Inc. M. Matsumizu, Y.

Nakazuru, A. Okayama, and M. Nagashima are employees of Pfizer Japan Inc.

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References

[1] Japanese Society for Sexually Transmitted Infections. Diagnosis and treatment guidelines for sexually transmitted diseases 2011 in Japan. Jpn J Sex Transm Infect 2011;22:87–91 [in Japanese].
[2] Sobel JD. Vulvovaginal candidosis. Lancet 2007;369:1961–71.
[3] Workowski KA, Berman S, Centers for Disease Control and Prevention (CDC). Sexually transmitted diseases treatment guidelines, 2010. MMWR Recomm Rep 2010;59:1–110.
[4] Gilbert DN, Moellering RC, Eliopoulos GM, Chambers HF, Saag MS, editors. The Sanford guide to antimicrobial therapy 2014. 44th ed. Antimicrobial Therapy, Inc.; 2014.
[5] Pitsouni E, Iavazzo C, Falagas ME. Itraconazole vs fluconazole for the treatment of uncomplicated acute vaginal and vulvovaginal candidiasis in nonpregnant women: a metaanalysis of randomized controlled trials. Am J Obstet Gynecol 2008;198:153–60.
[6] Tooley P. Preferences in the treatment of vaginal candidosis. Practitioner 1989;233:668–9.
[7] A comparison of single-dose oral fluconazole with 3-day intravaginal clotrimazole in the treatment of vaginal candidiasis. Report of an international multicentre trial. Br J Obstet Gynaecol 1989;96:226–32.
[8] Nurbhai M, Grimshaw J, Watson M, Bond C, Mollison J, Ludbrook A. Oral versus intra-vaginal imidazole and triazole anti-fungal treatment of uncomplicated vulvovaginal candidiasis (thrush). Cochrane Database Syst Rev 2007. Art. No.: CD002845, http://dx.doi.org/10.1002/14651858.CD002845.pub2.
[9] Mikamo H, Izumi K, Ito K, Tamaya T. Comparative study of the effectiveness of oral fluconazole and intravaginal clotrimazole in the treatment of vaginal candidiasis. Infect Dis Obstet Gynecol 1995;3:7–11.
[10] Mikamo H, Kawazoe K, Sato Y, Hayasaki Y, Tamaya T. Comparative study on the effectiveness of antifungal agents in different regimens against vaginal candidiasis. Chemotherapy 1998;44:364–8.
[11] De Punzio C, Garutti P, Mollica G, Nappi C, Piccoli R, Genazzani AR. Fluconazole 150 mg single dose versus itraconazole 200 mg per day for 3 days in the treatment of acute vaginal candidiasis: a double-blind randomized study. Eur J Obstet Gynecol Reprod Biol 2003;106:193–7.
[12] Rees T, Phillips R. Multicenter comparison of one-day oral therapy with fluconazole or itraconazole in vaginal candidiasis. Int J Gynecol Obstet 1992;37:33–8.
[13] Mizuno S, Hashimoto M, Shimoya Y, Sagae S, Yajima A, Hamazaki Y, et al. A double-blind comparative study of the efficacy of one-day administration of oxiconazole tablets for the treatment of vulvovaginal candidiasis. World Obstet Gynecol (Sanfujinkanosekai) 1989;41:633–45 [in Japanese].
[14] Mizuno S, Hashimoto M, Shimoya Y, Sagae S, Yajima A, Hamazaki Y, et al. A double-blind comparative study of the efficacy of six-day administration of oxiconazole tablets for the treatment of vulvovaginal candidiasis. World Obstet Gynecol (Sanfujinkanosekai) 1989;41:713–25 [in Japanese].
[15] Eckert LO, Hawes SE, Stevens CE, Koutsky LA, Eschenbach DA, Holmes KK. Vulvovaginal candidiasis: clinical manifestations, risk factors, management algorithm. Obstet Gynecol 1998;92:757–65.
[16] Gunther LS, Martins HP, Gimenes F, Abreu AL, Consolaro ME, Svidzinski TI. Prevalence of *Candida albicans* and non-albicans isolates from vaginal secretions: comparative evaluation of colonization, vaginal candidiasis and recurrent vaginal candidiasis in diabetic and non-diabetic women. Sao Paulo Med J 2014;132:116–20.
[17] Goswami D, Goswami R, Banerjee U, Dadhwal V, Miglani S, Lattif AA, et al. Pattern of *Candida* species isolated from patients with diabetes mellitus and vulvovaginal candidiasis and their response to single dose oral fluconazole therapy. J Infect 2006;52:111–7.
[18] Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Fourth Inf Suppl 2012;32:1–28.
[19] European Committee on Antimicrobial Susceptibility Testing. Antifungal agents breakpoint tables for interpretation of MICs. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Antifungal_breakpoints_v_6.1.pdf; 2013.
[20] Kobayashi T, Muratani T. Drug susceptibilities of yeast-like fungi isolated from vaginal discharge (proceedings). Jpn J Sex Transm Infect 2012;23:64 [in Japanese].
[21] Mikamo S, Yamagishi Y. Trichomoniasis and genital candidiasis. Rinshofujinkasanka 2013;67:52–7 [in Japanese].

梅毒

愛知医科大学病院感染症科
愛知医科大学病院感染制御部　山岸　由佳　　三鴨　廣繁

【梅毒とは】

梅毒は *Treponema pallidum* subspecies *pallidum*（*T. pallidum*）による感染症である。*T. pallidum* は直径0.1～0.2 μm, 長さ10～20 μmの屈曲した6～14施転の規則的間隔を持つらせん状菌である。「*pallidum*」の名は暗視野顕微鏡で青い色彩として観察されることに由来する。現在でも *T. pallidum* の試験管内での培養は不可能とされている。発育の至適温度は16～18℃で，42℃，1時間の加熱で死滅する。感染経路は主に性交渉や類似の行為で感染する性感染症の一つである。稀に接触感染や輸血による感染もある。皮膚や粘膜の微少な傷から *T. pallidum* が侵入することで感染し，局所で増殖したのち，やがて血行性に全身に散布され，種々の症状を引き起こす全身性の慢性感染症である。

胎児が母体内で経胎盤的に感染した場合を先天梅毒とし，それ以外の感染症を後天梅毒とよぶ。

また，皮膚や臓器での梅毒による症状がみられるものを顕性梅毒，症状はみられないが梅毒血清反応が陽性であるものを無症候梅毒という。

【臨床症状】

一般的に第1期梅毒（感染から約3週間），第2期梅毒（感染3ヶ月～3年），早期潜伏梅毒・後期潜伏梅毒，晩期梅毒（第3期梅毒，第4期梅毒）の順に進行する。また，潜伏梅毒の場合は早期潜伏梅毒と後期潜伏性梅毒に分類されるが，前者は感染後1年以内，後者は1年以降の時期を指す。無治療の場合は梅毒症例の約3分の1が晩期梅毒に移行すると言われている。潜伏梅毒は皮膚症状を中心に症状の再燃がみられる場合がある[1]（図1）。合併症として，HIVなどのその他の感染症を合併していることがあり，それを念頭においた診療が望まれる。

第1期梅毒（硬性下疳期）

T. pallidum に感染後，第一潜伏期（平均24日）を経て，皮膚や粘膜の *T. pallidum* 侵入部位に特有の限局性病変がみられる。これを初期硬結という。小豆大～示指頭大で，軟骨様の硬さである[2]。初期硬結は外陰部およびその周辺にみられ，女性では大小陰唇が最多である。稀に複数認められることもある[3]。その後，表皮が剥離して時に潰瘍化し，辺縁が隆起して基底と区別可能となった病変を硬性下疳という。硬性下疳は，性器に硬結を伴う無痛性の皮膚潰瘍である。硬性下疳部からは *T. pallidum* が検出され，感染性がある。次いで鼠径部のリンパ節腫脹がみられ，これを無痛性横痃（おうげん）といい，通常3～4週間で自然治癒する。男性では冠状溝，包皮，亀頭部，女性では大小陰唇，子宮頸部にみられるが女性では気づかれにくい。頻度は低いが陰部外にもみられることがある。初期硬結や硬性下疳も次第に消失するが，瘢痕は数ヶ月残存する。治癒した頃から梅毒血清反応が陽性となることになる。

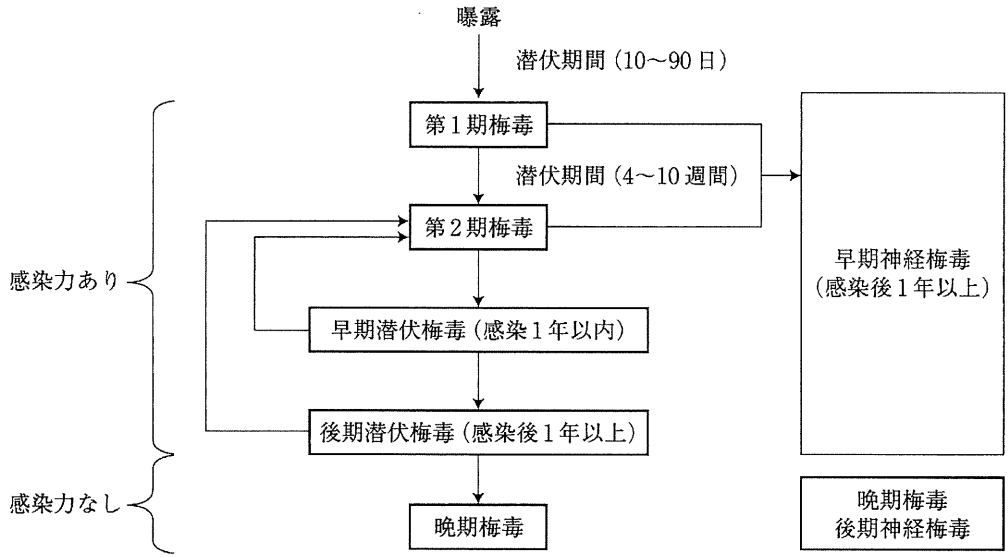


図1　梅毒の自然経過（文献1より）

第2期梅毒（バラ疹期）

皮膚粘膜病変が主な症状である。全身性にバラ疹といわれる皮疹がみられるほか，丘疹状や水疱状の多彩な皮疹がみられる。特に，手掌や足底の発疹は特徴的である。また，陰部や口腔粘膜に丘疹状の扁平コンジローマがみられ，脱毛もみられる。泌尿器系（糸球体腎炎，ネフローゼ症候群），中枢神経系（髄膜炎，脳神経障害，虹彩炎，ぶどう膜炎），筋骨格系（関節炎，骨炎，骨髄炎）にも症状を呈することがある。また発熱，全身倦怠感，全身性リンパ節腫大，関節痛，体重減少など，多彩な症状を呈することがある。この時期は *T. pallidum* の感染力が強く，血行性に全身の臓器へ播種する。

早期潜伏梅毒・後期潜伏梅毒

症状の再燃がみられる場合がある。

晩期梅毒

感染3年以上経過すると，結節性梅毒疹やゴム腫がみられることがある。10年以降にみられる症状では大動脈炎や大動脈瘤などがある。

神経梅毒

神経梅毒はこれまで晩期梅毒の症状と考えられていたが，*T. pallidum* は感染直後から中枢神経系に浸潤することが知られており[4]，早期から症状を呈することがある。臨床的には，早期神経梅毒と，後期神経梅毒に分類される。前者は，髄膜や脳神経に病変をきたすもので，無症候性髄膜炎，髄膜型，脳血管型などがある。後者は，主に脳や脊髄に病変をきたすもので，神経麻痺，脊髄癆などがある。それぞれの病名は複数が重なり合うことも多い。

無症候性髄膜炎は，神経学的な異常を認めないが，髄液検査で異常を認めるものをいう，多くは無症候性で，髄液細胞数は単球有意の100/μL未満，蛋白100mg/dL未満である[5]。髄膜型神経梅毒は，頭痛やけいれん，脳神経障害（視神経，聴神経，顔面神経など），精神障害を呈する。無熱が多く，髄液細胞数は200～400/μL，蛋白100～200mg/dLを示すことが多い[5]。脳血管型は，感染から5～12年後に

発症するとされており，広汎な髄膜の炎症とともに，脳動脈の炎症と繊維化による狭窄や閉塞がみられ，syphilitic apoplexy ともいわれる。髄液細胞数は 10 ～ 100/μL，蛋白は 100 ～ 200mg/dL を示すことが多い。後期神経梅毒のうち，神経麻痺は，多彩な精神症状が出現し，無治療では 5 年以内に死亡することが多い。脊髄癆は古典的には失調性歩行，疼痛，しびれ，膀胱機能障害，視神経萎縮による視力障害を主徴とする病態で，感染から 20 ～ 25 年を経過して発症する。Argyll Robertson 瞳孔，眼筋麻痺，Charcot 関節を呈することはよく知られている[5]。

先天梅毒

梅毒に罹患している母体から出生した児で，胎内感染，早期先天梅毒，後期先天梅毒の 3 つに分類される。感染は妊娠のどの時期でも起こり得るが，特に第 1 期，第 2 期は感染力が強く，未治療の場合の正常児出産率は約 20%，流・早産 40%，先天梅毒は 40%とされている[6]。日本国内では年 1 ～数名の報告がある。

胎内感染例では，生下時に老人性顔貌で，皮膚症状（全身性皮膚浸軟，梅毒性天疱瘡，証跡水疱形成など），その他（肝脾腫，脈絡網膜炎，骨軟骨炎など）の臨床症状がみられる。早期先天梅毒は，乳児梅毒ともいわれ，数週から 2 ～ 3 ヶ月で発症する。症状は，皮膚症状（赤色広範囲の浸潤，Parrot 裂溝，梅毒天疱瘡，爪床炎），その他（肝脾腫，哺乳不良，鞍鼻，嗄声，骨軟骨炎，それによる Parrot 仮性麻痺）など多彩である。後期先天梅毒は，学童期以降に発症し，皮膚症状（扁平コンジローマ，ゴム腫），その他の症状（実質性角膜炎，内耳性難聴，Hutchinson 歯といった Hutchinson 3 徴候，鞍鼻，脳脊髄障害，発作性ヘモグロビン尿などの症状）がみられる。

【診断と検査】

梅毒の診断には，血清学的検査（非トレポネーマ検査，特異的トレポネーマ検査）が主に用いられる。

非トレポネーマ検査はリン脂質のカルジオリピン抗原に対する抗体価を測定する serologic test for syphilis（STS）法で，rapid plasma regain card test（RPR）法，ラテックス凝集法が頻用されている。梅毒感染後 2 ～ 4 週間で陽性となり，通常第 2 期梅毒から早期潜伏梅毒にかけて最も高くなる。疾病の活動性と相関することが多いが，妊婦や高齢者，膠原病，慢性肝疾患，結核，HIV 感染などを有する場合に疑陽性となることがあるため結果の解釈には注意が必要である（生物学的偽陽性反応：biological false positive; BFP）。STS 法は梅毒の治療を開始すると値は低下するが，十分な治療を行っても抗体価が陰性にならない場合もある（serofast reaction）。なお，かつて用いられていたガラス板法は 2010 年に発売中止となっている。

特異的トレポネーマ検査は *T. pallidum* の菌体成分に対する反応を測定する方法（TP 抗原法）で，*Treponema pallidum* hemagglutination test（TPHA）法，fluorescent treponemal antibody absorption test（FTA-ABS）法，ラテックス凝集法，venereal disease research laboratry（VDRL）があるが，VDRL は日本では保険未収載である。特異的トレポネーマ検査は，通常は非トレポネーマ抗体が陽性となってから 2 ～ 3 週間後に遅れて陽性となる。非トレポネーマ抗体とは異なり疾患特異性が高く，陽性の場合にはこれまでに梅毒に曝露されたことを示し，確定診断には必須である。非トレポネーマ抗体のように疾患活動性とは相関せず，治療によって *T. pallidum* が消失した後も陽性が持続し，治療効果判定には使用されない。FTA-ABS 抗体のうち IgM 抗体は初感染後 1 週間で産生され約 1 ヶ月でピークに達し，その頃から IgG 抗体が産生されはじめ，3 ヶ月頃にピークに達するため，これらを組み合わせることでより正確な診断が可能となる。表 1 に妊婦および新生児における梅毒血清反応検査の結果と解釈を示す[7]。

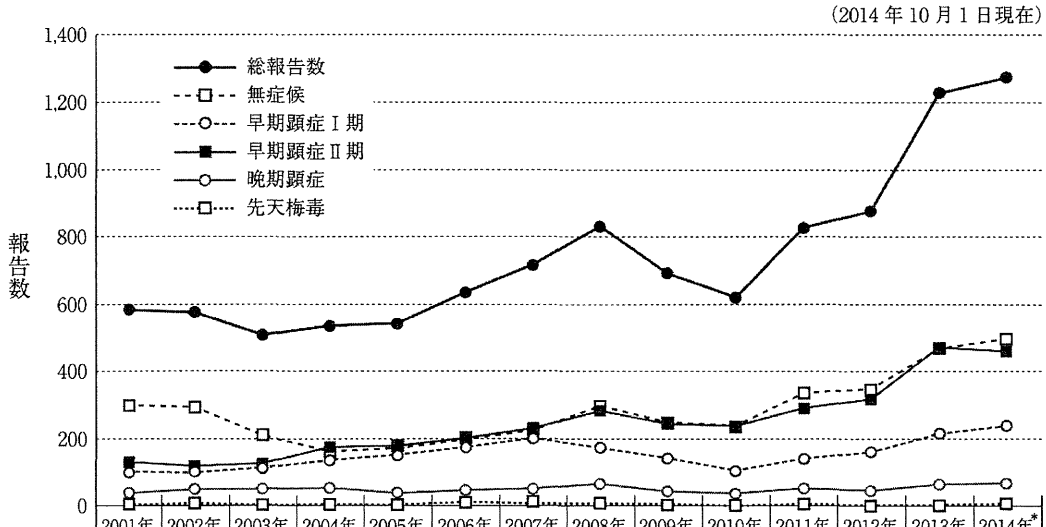
直接 *T. pallidum* を検出する方法として，初期硬結や硬性下疳の表面をメスで擦過して得た液体をスライドグラスに採取し，パーカー社製ブルー・ブラックインク，ギムザ液，または墨汁を混ぜて薄くのばし，乾燥後顕微鏡の油浸で観察または暗視野顕微鏡による観察法がある[8]。

表1　妊婦および新生児における梅毒血清反応の結果と解釈（文献7）

非トレポネーマ検査		トレポネーマ検査		解釈
母体	新生児	母体	新生児	
−	−	−	−	母体も児も梅毒ではない 感染直後またはプロゾーン現象
+	+／−	−	−	母体も児も梅毒 妊娠中に治療を受けた母体 母体は梅毒潜伏期，児も梅毒の可能性あり
+	+	+	+	母体は最近または少し前に梅毒に罹患 児も梅毒の可能性
−	−	+	+	母体は妊娠前，妊娠初期に梅毒治療に成功。 または偽陽性。児は梅毒とは考えにくい

【疫学】

日本における梅毒患者の推移を図2に示す[9]。かつての日本では1987年をピークに減少傾向を示していたが，最近の国立感染症研究所の感染症発生動向調査（IDWR）速報データによると，1999年の統計開始以来，2010年を境に感染報告数が過去最多のペースで伸びている[10]。最近では，都市部での感染者数が目立ち，さらに男性の感染が8割を占め，同性間の性的接触が男性間で特に多い。先天梅毒の報告例も年1～10人の報告が続いている[11]。



	2001年	2002年	2003年	2004年	2005年	2006年	2007年	2008年	2009年	2010年	2011年	2012年	2013年	2014年*
総報告数	585	575	509	536	543	637	718	831	691	621	828	875	1,228	1,275
無症候	301	293	209	160	172	195	224	300	249	238	335	348	466	497
早期顕症Ⅰ期	104	99	114	136	151	175	198	173	142	105	141	160	217	239
早期顕症Ⅱ期	134	121	127	179	180	205	234	283	251	236	292	315	475	460
晩期顕症	40	53	54	54	37	50	55	66	44	41	54	48	66	71
先天梅毒	6	9	5	7	3	12	7	9	5	1	6	4	4	8

＊2014年1月1日～10月1日（累積報告数）

図2　梅毒の年別・病型別報告数の推移（2001～2014年）（文献9より）

【法律】

梅毒は感染症法における第5類感染症に指定されているため，診断した医師は全例7日以内に都道府県知事に届出る必要がある[12]。感染後3週間で初期硬結を生じるが，その時点では梅毒血清反応は陰性であっても，病変部から *T. palidum* を暗視野顕微鏡などで直接検出するかその後の抗体価の推移で梅毒と診断したもの，カルジオリピンを抗原とする検査で16倍以上あるいはそれに相当する抗体を保有し，臨床的特徴を呈していないものや無症状のもの（無症状病原体保有者）は届け出る（表2）。ただし，陳旧性梅毒および梅毒治療後の単なる抗体保有者と見なされるものは届出る必要はない[12]。2010年にSTS法で届出基準となっていたガラス板法が2010年に発売中止となったことを受けて，ラテックス凝集法を用いた自動化法の基準が設けられ，当面は16.0 R.U. または16.0 S.U. 以上が記載されている。

表2　感染症法に基づく梅毒届出基準（文献12より）

ア　患者（確定例）

医師は，(2) の臨床的特徴を有する者を診察した結果，症状や所見から梅毒が疑われ，かつ，次の表の左欄に掲げる検査方法により，梅毒患者と診断した場合には，法第12条第1項の規定による届出を7日以内に行わなければならない。

この場合において，検査材料は，同欄に掲げる検査方法の区分ごとに，それぞれ同表の右欄に定めるもののいずれかを用いること。

イ　無症状病原体保有者

医師は，診察した者が (2) の臨床的特徴を呈していないが，次の表の左下欄に掲げる検査方法により，抗体（カルジオリピンを抗原とするRPRカードテスト，凝集法若しくはガラス板法での検査で16倍以上又は自動化法での検査で概ね16.0 R.U.，16.0 U若しくは16.0 SU/ml以上のものをいう。）を保有する者で無症状病原体保有者とみなされるもの（陳旧性梅毒とみなされる者を除く。）を診断した場合には，法第12条第1項の規定による届出を7日以内に行わなければならない。

この場合において，検査材料は，同欄に掲げる検査方法の区分ごとに，それぞれ同表の右欄に定めるもののいずれかを用いること。

ウ　感染症死亡者の死体

医師は，(2) の臨床的特徴を有する死体を検案した結果，症状や所見から，梅毒が疑われ，かつ，次の表の左欄に掲げる検査方法により，梅毒により死亡したと判断した場合には，法第12条第1項の規定による届出を7日以内に行わなければならない。

この場合において，検査材料は，同欄に掲げる検査方法の区分ごとに，それぞれ同表の右欄に定めるもののいずれかを用いること。

検査方法	検査材料
墨汁法，ギムザ染色などの染色法による病原体の検出	発疹（初期硬結，硬性下疳，扁平コンジローマ，粘膜疹）
・以下の（1）と（2）の両方に該当する場合 (1) カルジオリピンを抗原とする以下のいずれかの検査で陽性 ・RPRカードテスト，凝集法，ガラス板法，自動化法 (2) *T. pallidum* を抗原とする以下のいずれかの検査で陽性 ・TPHA法，FTA-ABS法	血清

先天梅毒は，下記の5つのうち，いずれかの要件をみたすものである。

ア　母体の血清抗体価に比して，児の血清抗体価が著しく高い場合
イ　児の血清抗体価が移行抗体の推移から予想される値を高く超えて持続する場合
ウ　児の *T. pallidum* を抗原とするIgM抗体陽性
エ　早期先天梅毒の症状を呈する場合
オ　晩期先天梅毒の症状を呈する場合

【治療】

梅毒の治療では，耐性の報告がなく，殺菌的薬剤であるペニシリン系薬が推奨されている[7]。各種ガイドラインが推奨している治療レジメンを表3に示す[8,13]。治療薬の投与期間は梅毒の病期によって異なり，第1期は2～4週間，第2期は4～8週間，第3期以降は8～12週とされている。神経梅毒ではベンジルペニシリンカリウム1日200～400万単位を1日6回点滴静注で10～14日間投与する。米国ではベンザチンペニシリンの筋肉内注射が第一選択[14]であるが，日本では使用できない。

妊婦の治療は垂直感染の予防を考慮し妊娠週数にかかわらず治療を開始する。ペニシリン系薬は妊婦にも投与可能である。ペニシリンアレルギーの場合は脱感作を行って投与を試みることが推奨される。

表3　梅毒の治療（文献8, 12より）

ガイドライン	推奨される治療レジメン
日本性感染症学会ガイドライン2011，改訂版[7]	ベンジルペニシリンベンザチン1回40万単位，1日3回，内服 アモキシシリン1回500mg，1日3回，内服 アミノベンジルペニシリン1回500mg，1日3回，内服 ペニシリンアレルギーの場合 塩酸ミノサイクリンまたはドキシサイクリン1回100mg，1日2回 妊婦の場合 アセチルスピラマイシン200mg，1日6回，内服 投与期間：上記薬剤を第1期2～4週間，第2期4～8週間，第3期以降8～12週間 【神経梅毒】 ベンジルペニシリンカリウム1回200～400万単位，1日6回，点滴静注，10日～2週間 先天梅毒 ベンジルペニシリンカリウム　点滴静注（推奨用量記載なし）
JAID/JSC感染症治療ガイド2014[12]	【第一選択】 ベンジルペニシリンベンザチン経口1回40万単位，1日3回 アンピシリン経口1回500mg，1日3回 アモキシシリン経口1回500mg，1日3回 【第二選択】 ペニシリンアレルギーの場合 ミノサイクリン経口1回100mg，1日2回 妊婦の場合 スピラマイシン経口1回200mg，1日6回 投与期間：上記薬剤を第1期2～4週間，第2期4～8週間，第3期以降8～12週間 【顕性梅毒】 【無症候性梅毒】 感染後1年以上経過あるいは感染時期が不明の場合は上記薬剤を8～12週間 【神経梅毒】 ベンジルペニシリン　点滴静注1回200～400万単位，1日6回，10～14日間

【治療上の留意点と治療効果判定】

抗菌薬を開始すると，開始後数時間で *T. pallidum* が破壊されるため，24時間以内に，高熱，悪寒，頭痛，筋肉痛，発疹などの症状が出現することがあり，これはJarisch-Herxheimer現象といわれる。この現象は第1期梅毒の約半数，第2期梅毒のほとんどの症例で起こるとされている。対症療法で軽快することが多いが，この現象が治療薬の副作用によるものではないことを含め，治療開始の際には患者に事前に説明しておく。また妊婦にはこの反応で流早産をきたすことがあるためより十分な説明をして注意する。

梅毒の治療中は感染防止の観点から，性交渉は控えることが望ましい。

治療効果判定は，自動化法で，同一のSTS法定量法で抗体価を測定し，値が低下，最終的には陰性化あるいは安定化することを確認する。治療後半年を経過しても16倍以上を示す場合は治療が不十分であるか，再感染あるいはHIVとの共感染を考慮する。治療後は3～6ヶ月毎に同じ測定法で定量測定を行い，再燃がないことを確認する。4倍希釈以上の値の増加は再感染や再発を疑うのが一般的であるが正式な定義はない。治療を行った妊婦ではその後妊娠28～32週と分娩時にSTS法で測定し治療効果を判定する[13]。神経梅毒の効果判定には髄液の検査も用いる。

【感染対策】

感染者の血液，尿，精液や湿性開放創などの分泌物の取扱いには留意する。妊婦については，分娩様式は適応がない限り帝王切開を選択する必要はないとされており，授乳は母乳栄養可能である。産後の児の取扱いについては母児同室でよいが，共有の沐浴場を用いる場合は最後に行い，児の排泄物などの取扱いには留意する。特に治療後から24時間までは手袋の装着が必須である。

文　献

1) Golden MR, et al. Update on syphilis: resurgence of an old problem. JAMA. 2003, 290, 1510 – 1514.
2) 伊東秀記，他．梅毒―どのような臨床症状のときに疑うか，1冊でわかる性感染症．本田まりこ編．東京，文光堂，2009，60 – 65.
3) 楠本雅彦，他．実地医科のための梅毒の診断と治療：特に妊婦梅毒．金原出版，1971，9 – 19.
4) Collaet P, et al. Experimental rabbit syphilis. Br J Vener Dis. 1971, 47, 389 – 400.
5) Ghanem KG. REVIEW: Neurosyphilis: A historical perspective and review. CNS Neurosci Ther. 2010, 16, e157 – 168.
6) Mascola L, et al. Inadequate treatment of syphilis in pregnancy. Am J Obstet Gynecol. 1984, 150, 945 – 947
7) Sexually Transmitted Diseases 4th, ed. 2008, 1598.
8) 日本性感染症学会．ガイドライン2011の改訂．日本性感染症学会ガイドライン．2013，24，51 – 54.
9) 国立感染症研究所．〈注目すべき感染症〉梅毒2014年における報告数増加と疫学的特徴．IDWR. 2014，47．<http://www.nih.go.jp/niid/images/idwr/douko/2014d/img47/chumoku01.gif>
10) 国立感染症研究所の感染症発生動向調査（IDWR）速報データ．<http://www.nih.go.jp/niid/images/idwr/sokuho/idwr-2014/201430/2014-30-zensu.pdf>
11) 国立感染症研究所．梅毒2008～2014．IASR．2015，36，17 – 19.
12) 厚生労働省．感染症法に基づく医師及び獣医師の届出について．15. 梅毒．<http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou11/01-05-11.html>
13) JAID/JSC感染症治療ガイド・ガイドライン作成委員会．JAID/JSC感染症治療ガイド2014．日本感染症学会・日本化学療法学会．東京，ライフサイエンス出版，2014，236 – 238.
14) Workowski KA, et al. Transmitted Diseases Guidelines, 2010. MMWR Recomm Rep. 2010, 59 (RR-12), 1 – 110.

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Human leukocyte antigen class II DRB1*1302 allele protects against cervical cancer: At which step of multistage carcinogenesis?

Koji Matsumoto,[1] Hiroo Maeda,[2] Akinori Oki,[1] Naoyoshi Takatsuka,[3] Toshiharu Yasugi,[4] Reiko Furuta,[5] Ranko Hirata,[2] Akira Mitsuhashi,[6] Kei Kawana,[4] Takuma Fujii,[7] Takashi Iwata,[7] Yasuo Hirai,[8] Masatoshi Yokoyama,[9] Nobuo Yaegashi,[10] Yoh Watanabe,[11] Yutaka Nagai,[12] Hiroyuki Yoshikawa[1] and for the Japan HPV and Cervical Cancer (JHACC) Study Group

[1]Department of Obstetrics and Gynecology, University of Tsukuba, Tsukuba; [2]Department of Transfusion Medicine and Cell Therapy, Saitama Medical Center, Saitama Medical University, Saitama; [3]Department of Health Economics, Gifu University Graduate School of Medicine, Gifu; [4]Department of Obstetrics and Gynecology, University of Tokyo, Tokyo; [5]Department of Pathology, Cancer Institute, Japanese Foundation of Cancer Research, Tokyo; [6]Department of Reproductive Medicine, Graduate School of Medicine, Chiba University, Chiba; [7]Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo; [8]Departments of Gynecology and Cytopathology, Cancer Institute Hospital, Japanese Foundation of Cancer Research, Tokyo; [9]Department of Obstetrics and Gynecology, Faculty of Medicine, Saga University, Saga; [10]Department of Obstetrics and Gynecology, Tohoku University School of Medicine, Sendai; [11]Department of Obstetrics and Gynecology, Kinki University School of Medicine, Osaka; [12]Department of Obstetrics and Gynecology, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan

Key words

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Correspondence

Koji Matsumoto, Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tsukuba, Tsukuba 305-8575, Japan.
Tel: +81 29 853 3073; Fax: +81 29 853 3072;
E-mail: matsumok@mui.biglobe.ne.jp

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We investigated the role of human leukocyte antigen (HLA) class II alleles in multistage cervical carcinogenesis. Cross-sectional analysis for HLA association with cervical cancer included 1253 Japanese women: normal cytology (NL, *n* = 341), cervical intraepithelial neoplasia grade 1 (CIN1, *n* = 505), CIN grade 2 or 3 (CIN2/3, *n* = 96), or invasive cervical cancer (ICC, *n* = 311). The HLA class II allele frequencies were compared by Fisher's exact test or the χ^2-test. The Bonferroni adjustment corrected for multiple comparisons. Among the study subjects, 454 women with low-grade squamous intraepithelial lesion cytology were prospectively monitored by cytology and colposcopy every 3–4 months to analyze cumulative risk of CIN3 within the next 10 years in relation to HLA class II alleles. HLA class II DRB1*1302 allele frequency was similar between women with NL (11.7%) and CIN1 (11.9%), but significantly decreased to 5.2% for CIN2/3 and 5.8% for ICC (*P* = 0.0003). Correction for multiple testing did not change this finding. In women with low-grade squamous intraepithelial lesion cytology, the cumulative risk of CIN3 diagnosed within 10 years was significantly reduced among DRB1*1302-positive women (3.2% *vs.* 23.7%, *P* = 0.03). In conclusion, the two different types of analysis in this single study showed the protective effect of the DRB1*1302 allele against progression from CIN1 to CIN2/3.

Cervical cancer is the third most common cancer in women worldwide, with approximately 530 000 women developing the disease every year. [1] Virtually all cases of cervical cancer are caused by persistent infection with carcinogenic HPVs, specifically, HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.[2] However, although carcinogenic HPV infection is common among young women of reproductive age, only a small subset of infected women develop cervical cancer, implying the involvement of additional risk factors.[3] Environmental factors such as smoking, parity, and OC use have been suggested as relevant to cervical carcinogenesis in numerous case–control studies.[4]

Genetic linkage with cervical cancer has also been implied.[5] There is *a priori* biological plausibility supporting HLA involvement in the development of HPV-related cancer.[6] HLA molecules are responsible for the presentation of viral antigens to the host immune system, thus playing a central role in immune recognition and subsequent clearance of virus-infected cells. Therefore, genetic variations in HLA regions may influence the efficiency of HPV antigen presentation and condition the immune responsiveness to HPV infections.[7]

It is known that rabbit MHC class II genes are associated with malignant conversion of cottontail rabbit papillomavirus-induced tumors.[8] To date, epidemiological studies have suggested that HLA class II DRB1*1501 and DQB1*03 may be associated with an increased risk of cervical cancer, whereas DRB1*13 may protect against cervical carcinogenesis.[7] However, results from these studies have not been entirely consistent. In addition, very little is known about the step at which HLA class II alleles may play a central role in multistep cervical cancer pathogenesis from HPV infection to cancer development because most HLA data on cervical cancer are based on case–control studies comparing HLA class II allele frequencies between cancer patients and healthy controls. There are few prospective studies addressing HLA

association with the development of cervical cancer and precancer.

In the present study, we analyzed HLA class II data from 1253 women with normal cytology, cervical precursor lesions, or invasive cervical cancer by using a cross-sectional study design. To reduce the risk of chance findings, putative links between cervical cancer and specific HLA class II alleles were also examined in a prospective cohort study of 454 women with low-grade cervical lesions. By using cross-sectional and prospective study designs, we investigated the protective effect of the HLA class II DRB1*1302 allele against progression to cervical precancer.

Materials and Methods

Study design. Our study subjects consisted of 1253 Japanese women (341 with NL, 505 with CIN1, 86 with CIN2, 10 with CIN3, and 311 with ICC) who visited 10 hospitals for cervical cancer screening, treatment of cervical diseases, or other reasons between April 1998 and March 2011. These women were included in the cross-sectional HLA class II analysis. Non-Japanese women were excluded from the present study based on self-reported ethnicity. At enrolment, blood samples were collected for HLA genotyping. Histological specimens were stained with H&E and reviewed by two pathologists (R. F. [author] and Tomoyuki Kitagawa [Department of Pathology, Cancer Institute Hospital, Japanese Foundation of Cancer Research, Tokyo, Japan]).

Of the study subjects included in the cross-sectional analysis, 591 women with CIN1 or CIN2 were followed at 3–4-month intervals and received cytology and colposcopic examinations at each visit. Of these, 454 women with evident LSIL cytology at baseline were included in the prospective analysis. This prospective cohort study has been described elsewhere.[9] Briefly, baseline cytology was reviewed by two cytopathologists (Y. H. [author] and Masafumi Tsuzuku [Department of Cytopathology, Cancer Institute Hospital, Japanese Foundation of Cancer Research]). At the time of study entry, cervical HPV DNA was determined by PCR-based methodology. In addition, information about smoking, contraceptive and reproductive history, and sexual behavior was also obtained from a self-administered questionnaire. During follow-up, a cervical biopsy was carried out only when Pap smears and colposcopic findings were suggestive of progression to CIN3 or worse.

For women whose condition was regarded as progressing, based on cytology and histology examinations carried out in the participating hospitals, the two cytopathologists and two pathologists reviewed all cytological and histological specimens collected for diagnosis of disease progression. In the prospective study, the primary endpoint was histological CIN3 lesions or worse diagnosed after rigorous pathological review. Occasionally a few difficult cases were adjudicated by joint review with consideration of cytology as well as histology. We used the primary endpoint of CIN3 or worse because CIN3 is a more certain, rigorous histological diagnosis of precancer than CIN2.[3]

Women entered the study voluntarily only after giving their signed informed consent. The study protocol was approved by the ethical and research review boards of the participating institutions.

Genotyping of HLA class II. Blood leukocytes were used for HLA genotyping. Total cellular DNA was extracted from these specimens and amplified by PCR using locus-specific primers. All samples were initially typed at the HLA-DRB1 and DQB1 loci using a commercially available reverse sequence-specific oligonucleotide probe typing kit (Dynal RELI SSO; Dynal Biotech, Oslo, Norway). For subtyping, group-specific amplifications were carried out as previously described.[10] DRB1 and DQB1 alleles were identified by SSCP and RFLP using the PCR products. Laboratory staff who carried out the HLA class II typing were blinded to the clinical data collected from the study subjects.

Genotyping of HPV. We detected HPV DNA in cervical samples by PCR-based methodology as described previously.[11] In brief, HPV DNA was amplified by PCR using consensus primers (L1C1 / L1C2 + L1C2M) for the HPV L1 region. The HPV types were identified by RFLP that has been shown to identify at least 26 types of genital HPVs.[12] Laboratory staff who carried out HPV genotyping were blinded to the clinical data collected from the study subjects.

Statistical analysis. In the cross-sectional study, HLA class II allele frequencies were compared by Fisher's exact test or the χ^2-test. When an expected cell value in the 2×2 tables was <5, Fisher's exact test was used. We also analyzed the data using the Bonferroni adjustment, a conventional method of correcting for multiple comparisons.

In the prospective cohort study, time to event was measured from the date of the index visit (i.e., the first instance of an abnormal cytology result) to the date of the visit at which cytological transition to CIN3 was first detected. Women whose lesions did not progress to CIN3 were censored at their last recorded return visit dates. The cumulative probability of progression to CIN3 was estimated using the Kaplan–Meier method and compared with a log–rank test, and the Cox regression model was used for statistical adjustments. Patient age, CIN grade, at the time of entry, HPV risk category (HPV16/18/31/33/35/45/52/58, other carcinogenic types, or carcinogenic HPV negative), smoking status, parity, OC use, number of lifetime sexual partners, and age at first sexual intercourse were included in the multivariate model for adjustments. As the results did not differ among the 10 hospitals, the study sites were not included in the multivariate models.

All analyses were carried out using the STATA 9 (StataCorp LP, College Station, TX, USA) statistics package. Two-sided *P*-values were calculated throughout and considered to be significant at <0.05.

Results

Cross-sectional analysis. The distribution of HLA-DRB1 and HLA-DQB1 alleles in 1253 Japanese women with normal cytology or cervical diseases is shown in Table 1. To examine the step at which HLA class II alleles may contribute to cervical carcinogenesis, the HLA data were compared among four groups: NL (n = 341), CIN1 (n = 505), CIN2/3 (n = 96), and ICC (n = 311).

We could not find any association between HLA class II alleles and development of CIN1, because HLA class II allele frequencies were very similar between women with NL and CIN1 (Table 1). DRB1*0901 frequency was significantly decreased among women with CIN2 or worse (CIN2+) compared with women with NL or CIN1 (<CIN2) (15.0% *vs.* 26.2%; P = 0.0003; Pc = 0.007). In addition, DRB1*1302 frequency was also significantly lower among women with CIN2 + (5.9% *vs.* 11.2%, P = 0.0003, Pc = 0.007). Although the number of women with CIN2/3 was small (n = 96), DRB1*0901 frequency was significantly decreased in women with ICC compared with women with CIN2/3 (14.5% *vs.*

Table 1. Correlation between human leukocyte antigen class II carrier frequencies and cervical diseases in Japanese women (n = 1253) at the commencement of the study

	NL		CIN1		CIN2/3		ICC		Relative risk of CIN2 or worse†		Correction for multiple comparison
	n = 341	%	n = 505	%	n = 96	%	n = 311	%	OR (95% CI)	P-value	Corrected P-value‡
DRB1*											
0101	30	9	57	11	8	8	33	11	0.98 (0.66–1.45)	0.91	
0401	9	3	5	1	3	3	12	4	2.27 (1.09–4.76)	0.03	0.72
0403	25	7	27	5	8	8	18	6	1.04 (0.64–1.69)	0.87	
0405	91	27	107	21	18	19	68	22	0.87 (0.66–1.17)	0.37	
0406	17	5	25	5	4	4	10	3	0.68 (0.37–1.26)	0.21	
0407	5	1	6	1	0	0	1	0	0.19 (0.02–1.45)	0.12	
0410	16	5	32	6	4	4	18	6	0.95 (0.57–1.60)	0.85	
0701	4	1	3	1	2	2	5	2	2.10 (0.73–6.02)	0.17	
0802	28	8	44	9	9	9	24	8	0.95 (0.62–1.49)	0.81	
0803	45	13	92	18	16	17	48	15	0.97 (0.70–1.33)	0.83	
0901	90	26	136	27	27	28	45	14	**0.59 (0.44–0.79)**	**0.0003§**	**0.007**
1001	3	1	10	2	1	1	5	2	0.96 (0.36–2.54)	0.93	
1101	18	5	26	5	6	6	14	5	0.94 (0.54–1.62)	0.83	
1201	22	6	22	4	4	4	17	5	0.99 (0.58–1.69)	0.98	
1202	9	3	13	3	1	1	4	1	0.47 (0.18–1.24)	0.15	
1301	2	1	5	1	3	3	1	0	1.19 (0.35–4.09)	0.75	
1302	40	12	60	12	5	5	18	6	**0.44 (0.28–0.71)**	**0.0003§**	**0.007**
1401	33	10	36	7	9	9	22	7	0.92 (0.60–1.44)	0.74	
1403	12	4	18	4	4	4	10	3	0.97 (0.51–1.85)	0.92	
1405	11	3	18	4	7	7	14	5	1.52 (0.86–2.72)	0.15	
1406	10	3	16	3	3	3	11	4	1.12 (0.58–2.18)	0.73	
1501	46	13	84	17	18	19	48	15	1.07 (0.77–1.47)	0.70	
1502	76	22	115	23	25	26	91	29	1.37 (1.04–1.79)	0.02	0.48
1602	5	1	7	1	2	2	1	0	0.52 (0.14–1.84)	0.41	
DQB1*											
0202	2	1	2	0	1	1	3	1	2.09 (0.51–8.40)	0.28	
03	203	60	282	56	58	60	176	57	1.01 (0.79–1.28)	0.96	
0301	77	23	94	19	21	22	56	18	0.92 (0.68–1.24)	0.59	
0302	60	18	86	17	15	16	50	16	0.91 (0.66–1.25)	0.57	
03032	96	28	138	27	31	32	86	28	1.06 (0.81–1.37)	0.69	
0401	89	26	105	21	18	19	66	21	0.87 (0.65–1.17)	0.35	
0402	29	9	55	11	10	10	29	9	0.96 (0.64–1.43)	0.85	
0501	33	10	66	13	9	9	37	12	0.96 (0.66–1.39)	0.84	
0502	24	7	24	5	6	6	14	5	0.86 (0.52–1.47)	0.58	
05031	26	8	41	8	8	8	29	9	1.16 (0.76–1.77)	0.48	
0601	110	32	189	37	40	42	134	43	1.37 (1.07–1.74)	0.01	0.24
0602	42	12	80	16	17	18	46	15	1.09 (0.78–1.51)	0.62	
0603	2	1	5	1	3	3	1	0	1.19 (0.35–4.09)	0.78	
0604	39	11	59	12	5	5	16	5	**0.41 (0.26–0.68)**	**0.0001§**	**0.001**

†Relative risks of cervical intraepithelial neoplasia grade 2/3 (CIN2/3)/invasive cervical cancer (ICC) were analyzed in comparison with <CIN2 (normal cytology or CIN1, n = 846). ‡P-values were calculated by Bonferroni adjustment to correct for multiple comparison. §Bold letters indicate statistical significance. CI, confidence interval; NL, normal cytology; OR, odds ratio.

28.1%; P = 0.003). DRB1*1302 frequency was similar between women with ICC and CIN2/3 (5.8% *vs.* 5.2%; P = 0.99).

DQB1*0604 frequency was also significantly decreased among women with CIN2+ compared to those with <CIN2 (5.2% *vs.* 11.6%; P = 0.0001; Pc = 0.001).

In the cross-sectional analysis, we could not detect any significant associations between other HLA class II alleles and development of CIN2 + (Table 1). The DRB1*0401, DRB1*1502, and DQB1*0601 allele frequencies were significantly associated with an increased risk of CIN2 + (P = 0.03, P = 0.02, and P = 0.01, respectively), but these associations lost significance after correction for multiple comparison. Although DRB1*1501 and DQB1*03 alleles have been suggested as relevant to an increased risk of cervical cancer in previous HLA studies,[7] the frequencies of these alleles were similar between women with <CIN2 and CIN2 + (P = 0.99 and P = 0.95, respectively). Another analysis comparing HLA allele frequencies between women with and without ICC did not change the findings (data not shown).

Prospective analysis. The clinical outcomes of 454 women with LSIL cytology were monitored by cytologic and colposcopic testing at intervals of 3–4 months. Table 2 shows the characteristics of the study subjects included in the prospective analysis. At baseline, 407 women had biopsy-proven CIN1 and 47 had biopsy results of CIN2. The mean age of the study sub-

Table 2. Characteristics of Japanese women with low-grade squamous intraepithelial lesion cytology (n = 454) included in prospective analysis

	All study subjects (n = 454), n (%)	DRB1*1302 status		P-value
		Positive (n = 47), n (%)	Negative (n = 407), n (%)	
Age, years				
18–29	86 (18.9)	7 (14.9)	79 (19.4)	0.13
30–39	217 (47.8)	18 (38.3)	199 (48.9)	
40+	151 (33.3)	22 (46.8)	129 (31.7)	
Histology at entry				
CIN grade 1	389 (85.7)	44 (94)	345 (85)	0.10
CIN grade 2	65 (14.3)	3 (6)	62 (15)	
HPV genotypes				
HPV16, 18, 31, 33, 35, 45, 52, 58	201 (44.3)	19 (40.4)	182 (44.7)	0.49
HPV39, 51, 56, 59, 68	110 (24.2)	17 (36.2)	93 (22.9)	
Low-risk types or negative	67 (14.8)	4 (8.5)	63 (15.5)	
Undetermined	32 (7.0)	5 (10.6)	27 (6.6)	
Multiple infection	38 (8.4)	2 (4.2)	36 (8.8)	
Parity				
0	151 (33.3)	15 (31.9)	136 (30.4)	0.50
1–2	243 (53.5)	28 (59.6)	215 (52.8)	
3+	60 (13.2)	4 (8.5)	56 (13.8)	
Use of oral contraceptives				
Yes	39 (8.6)	3 (6.7)	36 (8.8)	0.49
No	378 (90.6)	42 (93.3)	336 (82.6)	
Unknown	37 (8.1)	4 (8.5)	33 (8.1)	
Smoking				
Never smokers	209 (46.0)	15 (31.9)	185 (45.5)	0.89
Smokers	210 (46.3)	30 (63.8)	189 (46.4)	
Current smokers	151 (33.3)	24 (51.1)	136 (33.4)	
Former smokers	59 (13.0)	6 (12.8)	53 (14.2)	
Unknown	35 (7.8)	2 (4.3)	33 (8.1)	
Number of lifetime sexual partners				
1	68 (15.0)	6 (12.8)	62 (15.2)	0.84
2–3	115 (25.3)	13 (27.7)	102 (25.1)	
4	235 (51.8)	26 (55.3)	209 (51.4)	
Unknown	36 (7.9)	2 (4.3)	34 (8.4)	
Age at first sexual intercourse, years				
≤20	150 (33.0)	16 (34.0)	134 (32.9)	0.72
21–23	176 (38.8)	17 (36.2)	159 (39.1)	
≥24	93 (20.5)	12 (25.5)	81 (19.9)	
Unknown	35 (7.7)	2 (4.3)	33 (8.1)	

CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.

jects was 35.9 years (range, 19–54 years). In the current study, we updated the previous cohort data and extended the mean follow-up period from 39.0 months (range, 6.8–84.9 months) to 62.7 months (range, 6.8–155.9 months). By updating the data, the number of women diagnosed as progressing to CIN3 over the period of follow-up increased from 39 to 61.

Unlike the cross-sectional analysis results, DRB1*0901 was not associated with progression to CIN3 in the prospective analysis (P = 0.88; Fig. 1a). Adjustment for age, CIN grade at the time of entry, HPV risk category, parity, smoking status, OC use, number of lifetime sexual partners, and age at first sexual intercourse did not change this finding (P = 0.71; Table 3).

The risk of progression to CIN3 was significantly reduced among DRB1*1302-positive women (P = 0.03, log–rank test; Fig. 1b), which was consistent with the cross-sectional analysis results. This protective effect of DRB1*1302 against progression to CIN3 remained statistically significant, even after adjustment for possible risk factors of cervical cancer (adjusted P = 0.04; Table 3). Distributions of baseline characteristics among the DRB1*1302-positive and DRB1*1302-negative women are presented in Table 2. Because of the poor reproducibility of cervical histologic interpretations,[13,14] some CIN3 lesions may have been classified incorrectly as CIN2 at baseline. Therefore, we also analyzed the follow-up data for CIN1 and CIN2 separately. In women with CIN1 histology (n = 407), the cumulative risk of CIN3 diagnosed within 10 years was lower among DRB1*1302-positive women (3.5% *vs.* 17.6%; P = 0.09). In women with CIN2 histology (n = 47), progression to CIN3 did not occur in DRB1*1302-positive women (cumulative probability of histological CIN3 diagnosis within 10 years, 0.0% *vs.* 48.9%; P = 0.28). Although these associations did not reach statistical significance due to limitations imposed by the small sample size, similar patterns were observed in separate analyses for CIN1 and CIN2.

We analyzed the protective effect of DRB1*1302 allele in relation to HPV type-specific risk category. Among DRB1*1302-positive women, only one case positive for HPV33 progressed to CIN3. Therefore, in women positive for HPV16, 18, 31, 33, 35, 45, 52, or 58, the cumulative risk of CIN3 diagnosed within 10 years was 5.9% for those who were DRB1*1302-positive and 39.6% for those who were DRB1*1302-negative (P = 0.04, log–rank test). In women positive for HPV39, 51, 56, 59, or 68, the CIN3 risk within 10 years was 0.0% for those who were DRB1*1302-positive and 9.6% for those who were DRB1*1302-negative (P = 0.29, log–rank test). In women negative for high-risk HPVs, the CIN3 risk was 0.0% for DRB1*1302-positives and 1.9% for DRB1*1302-negatives (P = 0.78).

The DQB1*0604 allele was closely linked to the DRB1*1302 allele, indicating a strong correlation (r^2 = 0.96). As the DRB1*1302 and DQB1*0604 alleles were in linkage disequilibrium, similar findings were observed for the DRB1*1302 and DQB1*0604 alleles (Table 3). Therefore, only DRB1*1302 data are shown in Fig. 1.

We could not find any significant association between other HLA class II alleles and progression to CIN3. Data are only shown on two representative HLA class II alleles (DRB1*1501 and DQB1*03) that have been reported to be a risk factor for cervical cancer in previous HLA studies.[7] In the present study, these alleles did not affect the risk of progression to CIN3 within the next 10 years (Fig. 1c,d). Adjustment for possible cervical cancer risk factors did not change these findings (Table 3).

Discussion

By using cross-sectional and prospective study designs, we indicated the protective effect of the DRB1*1302 allele against progression to cervical cancer and precancer. The consistent results obtained by two different analyses appear to provide

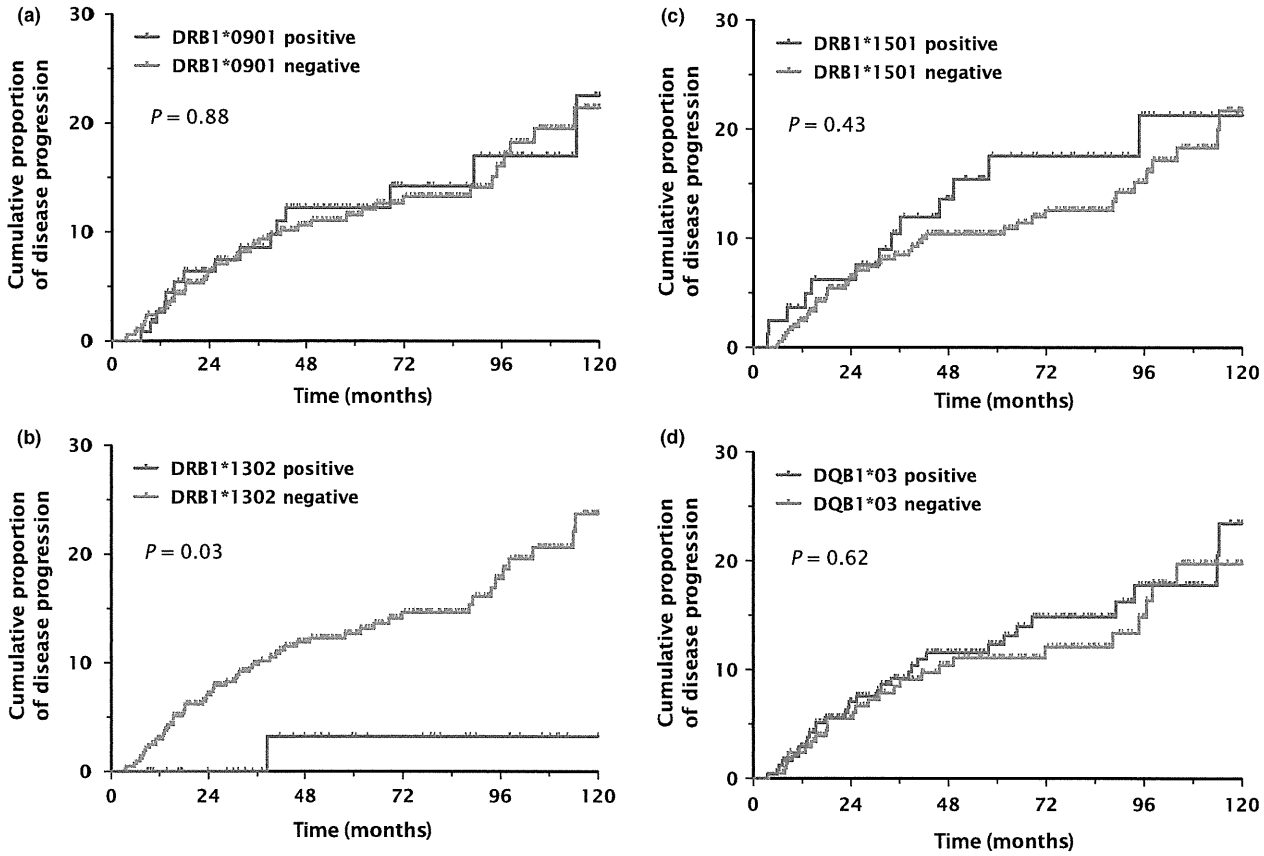

Fig. 1. Cumulative risks of cervical intraepithelial neoplasia grade 3 within 10 years in relation to human leukocyte antigen class II polymorphisms. A Kaplan–Meier plot was used to estimate the cumulative 10-year probabilities of progression to cervical intraepithelial neoplasia grade 3 among women with low-grade squamous intraepithelial lesion cytology for selected human leukocyte antigen class II alleles: DRB1*0901 (a), DRB1*1302 (b), DRB1*1501 (c), and DQB1*03 alleles (d). *P*-values were calculated using the log–rank test.

stronger evidence for the protective effect of the DRB1*1302 allele. In addition, negative associations between the DRB1*13 alleles and cervical cancer have been consistently reported in case–control studies.[7] In a small prospective study of French women with CIN1 (n = 86), a relationship between the DRB1*13 alleles and cytological regression was observed.[15] These observations also support the protective effect of the DRB1*13 alleles. However, some studies have reported a protective effect of DRB1*1301,[16,17] and other studies have suggested that DRB1*1302 decreases the risk of cervical cancer.[18–19] In the present study, we confirmed only the protective effect of the DRB1*1302 allele because the DRB1*1301 allele is rarely detected in Japanese populations.

Cervical cancer arises by way of several carcinogenic steps: HPV acquisition, HPV persistence (development of low-grade cervical precursor lesion), progression of a persisting infection to cervical precancer, and invasion through the basement membrane of the epithelium.[3,20] In multistage cervical carcinogenesis, however, the step at which DRB1*13 alleles exert their protective effect is not fully understood. Two prospective studies of HPV infections have shown that DRB1*13 alleles do not play a protective role against the acquisition and persistence of viral infections.[21,22] This is consistent with our finding that the DRB1*1302 frequency was similar between women with NL (12%) and CIN1 (11%). In the present study, the DRB1*1302 frequency significantly decreased to 5.2% for CIN2/3 and 5.8% for ICC. In the prospective analysis of women with cytological LSIL and histological CIN1/2, the cumulative probability of CIN3 diagnosed within the next 10 years was significantly low among DRB1*1302-positive women. These observations suggest that the DRB1*1302 allele may act protectively against progression from CIN1 to CIN2/3. Several studies have reported the poor reproducibility of CIN grading, even among well-trained observers.[13,14] In particular, CIN2 is an equivocal diagnosis of precancer (a heterologous borderline category between CIN1 and CIN3).[3] Therefore, it may be difficult to strictly determine whether the DRB1*1302 allele protects against progression from CIN1 to CIN2, or from CIN2 to CIN3. The protective effect of the DRB1*1302 allele against progression to cervical precancer suggests that DRB1*13 alleles could contribute to the immunological recognition of viral antigens such as the E7 protein, which is increasingly expressed during progression to cervical precancer.[23,24] However, very little is known about the binding of DRB1*13 molecules to HPV-related antigens.

As the DRB1*1302 and DQB1*0604 alleles were in linkage disequilibrium, very similar findings were observed for the DRB1*1302 and DQB1*0604 alleles in both cross-sectional and prospective analyses. Although the protective effect of DRB1*13 alleles is the most consistent HLA finding in

Table 3. Effect of selected human leukocyte antigen (HLA) class II alleles on progression to cervical intraepithelial neoplasia grade 3 in Japanese women with low-grade squamous intraepithelial lesion (n=454)

HLA class II alleles	*n*	Person-months	Events	10-year progression rate (95% CI)	Log–rank test	Adjusted analysis†	
					P-value	Hazard ratio (95% CI)	*P*-value
DRB1*0901							
Positive	118	7274.9	15	22.5 (11.9–38.6)	0.88	1.15 (0.53–2.33)	0.71
Negative	336	20 715.1	43	21.4 (15.2–29.4)		1.00	
DRB1*1302							
Positive	47	2916.7	1	3.2 (0.5–20.8)	0.03	0.13 (0.02–0.94)	0.04
Negative	407	25 073.3	57	23.7 (17.6–31.5)		1.00	
DRB1*1501							
Positive	82	5163.5	13	21.3 (12.3–35.5)	0.43	1.12 (0.56–2.425	0.76
Negative	372	22 826.5	45	21.8 (15.5–30.1)		1.00	
DQB1*03							
Positive	244	14 438.9	32	23.5 (15.3–35.0)	0.62	1.26 (0.72–2.20)	0.41
Negative	210	13 551.1	26	19.8 (13.1–29.2)		1.00	
DQB1*0604							
Positive	46	2879.6	1	3.2 (0.5–20.8)	0.03	0.13 (0.02–0.95)	0.04
Negative	408	25 110.4	57	23.7 (17.6–31.4)		1.00	

†Cox regression model was used for statistical adjustments. Patient age, histological grade at the time of entry, human papillomavirus (HPV) risk category (HPV16/18/31/33/35/45/52/58, other carcinogenic types, or carcinogenic HPV negative), smoking status, parity, use of oral contraceptives, number of lifetime sexual partners, and age at first sexual intercourse were included in the multivariate model for adjustments. CI, confidence interval.

published reports, the question is still open as to whether DRB1*1302 alone, DQB1*0604 alone, or both are associated with reduced risk of cervical diseases, or whether other MHC complex genes in linkage disequilibrium with these alleles are more important. To evaluate the independent effect of the DRB1*1302 and DQB1*0604 alleles, larger studies will be required.

The cross-sectional analysis suggested that the DRB1*0901 allele may act protectively against cervical cancer pathogenesis. A meta-analysis also reported the protective effect of DRB1*0901 against invasive cervical squamous cell carcinoma in Caucasian populations.[25] Interestingly, this allele was not associated with progression to CIN3 in the prospective analysis, suggesting that the DRB1*0901 allele could exert a protective effect on progression from CIN2/3 to ICC. The DRB1*0901 allele frequency was significantly lower in women with ICC than in those with CIN2/3. Alternatively or additionally, the discrepancy may be explained by the HPV type-specific effect of the DRB1*0901 allele. When the analysis was confined to HPV16-positive women with LSIL cytology, the cumulative probability of progression to CIN3 was lower among DRB1*0901-positive women compared with DRB1*0901-negative women (data not shown), but this effect was not statistically significant ($P = 0.12$) due to limitations imposed by the small sample size.

We could not find any significant association between DRB1*1501 or DQB1*03 and risk of developing CIN2/3 or ICC. Effects of the DRB1*1501 and DQB1*03 alleles have not been found in prospective cohort studies so far,[15,21,22] and the results from case–control studies have not been entirely consistent.[7,16–19,25–27] Although several groups have suggested a significant association of DRB1*1501 or DQB1*0602 (or the corresponding haplotype DRB1*1501-DQB1*0602) with HPV16-positive cervical cancer,[17,26,27] an increased risk of progression to CIN3 was not observed for either the DRB1*1501 or DQB1*0602 allele, even among HPV16-positive women in the present study (data not shown). The inconsistent results regarding the DRB1*1501 and DQB1*0602 alleles may be explained by two studies suggesting that cancer risks associated with these alleles may vary according to HPV16 E6 variations.[27,28]

One may speculate that HLA class II DRB1*13 testing, alone or in combination with genotype-specific HPV testing, for women with low-grade cervical abnormalities might be useful for identifying populations at decreased risk of disease progression. Interestingly, the protective effect of the DRB1*1302 allele appears to be non-specific to the HPV genotype. However, HLA class II testing may not be recommended in clinical practice because only 10–20% of women among various ethnic populations have the DRB1*13 alleles.[7] In addition, DRB1*13 does not prevent all cervical cancer; some women with cervical cancer are positive for DRB1*13.

The present study had several limitations. First, possible misclassification of CIN lesions may have affected the results. Recently, p16INK4a immunohistochemistry has been shown to increase the sensitivity and specificity of CIN2–3 detection in cervical biopsies.[29] Although two pathologists reviewed all histological specimens, the histological diagnosis of cervical specimens was obtained by H&E examination alone. Second, the present study may have missed several HLA associations with cervical diseases due to limitations imposed by the small sample size. Although this is the first large-scale study on HLA association with cervical cancer in Japan, larger studies will be required to further evaluate the risk of cervical cancer and precancer in relation to HLA polymorphism. Finally, the present study did not clarify the mechanism by which the DRB1*1302 allele protects against cervical precancer. To address this, immunological studies on the binding of

DRB1*13 molecules to HPV-related antigens will be required. Analyses of other genes in the MHC complex found in linkage disequilibrium with the DRB1*13 alleles may also give insight to this protective effect.

In conclusion, our results confirmed the protective effects of DRB1*1302 through two different types of analysis in a single study. Our data also suggested that the DRB1*1302 allele may function protectively against progression from CIN1 to CIN2/3. However, the mechanism underlying the protective effect of DRB1*1302 allele is not fully understood. To enhance our biological understanding of this effect, identification of specific viral epitopes presented by the DRB1*1302 allele will be needed.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

CIN1–3	cervical intraepithelial neoplasia grade 1–3
HLA	human leukocyte antigen
ICC	invasive cervical cancer
HPV	human papillomavirus
LSIL	low-grade squamous intraepithelial lesion
NL	normal cytology
OC	oral contraceptive
Pc	*P* corrected by the Bonferroni method

References

1 Ferlay J, Shin HR, Bray F *et al.* Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2008; **127**: 2893–917.
2 Bouvard V, Baan R, Straif K *et al.* WHO International Agency for Research on Cancer Monograph Working Group. A review of human carcinogens–Part B: biological agents. *Lancet Oncol* 2009; **10**: 321–2.
3 Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. *Lancet* 2007; **370**: 890–907.
4 Castellsagué X, Muñoz N. Chapter 3: cofactors in human papillomavirus carcinogenesis–role of parity, oral contraceptives, and tobacco smoking. *J Natl Cancer Inst Monogr* 2003; **31**: 20–8.
5 Magnusson PK, Sparén P, Gyllensten UB. Genetic link to cervical tumours. *Nature* 1999; **400**: 29–30.
6 Breitburd F, Ramoz N, Salmon J, Orth G. HLA control in the progression of human papillomavirus infections. *Semin Cancer Biol* 1996; **7**: 359–71.
7 Hildesheim A, Wang SS. Host and viral genetics and risk of cervical cancer: a review. *Virus Res* 2002; **89**: 229–40.
8 Han R, Breitburd F, Marche PN, Orth G. Linkage of regression and malignant conversion of rabbit viral papillomas to MHC class II genes. *Nature* 1992; **356**: 66–8.
9 Matsumoto K, Maeda H, Oki A, *et al.* for Japan HPV And Cervical Cancer (JHACC) Study Group. HLA class II DRB1*1302 allele protects against progression to cervical intraepithelial neoplasia grade 3: a multicenter prospective cohort study. *Int J Gynecol Cancer* 2012; **22**: 471–8.
10 Konno Y, Numaga J, Tsuchiya N *et al.* HLA-B27 subtypes and HLA class II alleles in Japanese patients with anterior uveitis. *Invest Ophthalmol Vis Sci* 1999; **40**: 1838–44.
11 Yoshikawa H, Kawana T, Kitagawa K *et al.* Detection and typing of multiple genital human papillomaviruses by DNA amplification with consensus primers. *Jpn J Cancer Res* 1991; **82**: 524–31.
12 Nagano H, Yoshikawa H, Kawana T *et al.* Association of multiple human papillomavirus types with vulvar neoplasias. *J Obstet Gynaecol Res* 1996; **22**: 1–8.
13 Stoler MH, Schiffman M; Atypical Squamous Cells of Undetermined Significance-Low-grade Squamous Intraepithelial Lesion Triage Study (ALTS) Group. Interobserver reproducibility of cervical cytologic and histologic interpretations: realistic estimates from the ASCUS-LSIL Triage Study. *JAMA* 2001; **285**: 1500–5.
14 Carreon JD, Sherman ME, Guillén D *et al.* CIN2 is a much less reproducible and less valid diagnosis than CIN3: results from a histological review of population-based cervical samples. *Int J Gynecol Pathol* 2007; **26**: 441–6.
15 Sastre-Garau X, Cartier I, Jourdan-Da Silva N *et al.* Regression of low-grade cervical intraepithelial neoplasia in patients with HLA-DRB1*13 genotype. *Obstet Gynecol* 2004; **104**: 751–5.
16 Wang SS, Wheeler CM, Hildesheim A *et al.* Human leukocyte antigen class I and II alleles and risk of cervical neoplasia: results from a population-based study in Costa Rica. *J Infect Dis* 2001; **184**: 1310–14.
17 Beskow AH, Josefsson AM, Gyllensten UB. HLA class II alleles associated with infection by HPV16 in cervical cancer *in situ*. *Int J Cancer* 2001; **93**: 817–22.
18 Maciag PC, Schlecht NF, Souza PS *et al.* Major histocompatibility complex class II polymorphisms and risk of cervical cancer and human papillomavirus infection in Brazilian women. *Cancer Epidemiol Biomarkers Prev* 2000; **9**: 1183–91.
19 Madeleine MM, Johnson LG, Smith AG *et al.* Comprehensive analysis of HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 loci and squamous cell cervical cancer risk. *Cancer Res* 2008; **68**: 3532–9.
20 Scarinci IC, Garcia FA, Kobetz E *et al.* Cervical cancer prevention: new tools and old barriers. *Cancer* 2010; **116**: 2531–42.
21 Maciag PC, Schlecht NF, Souza PS *et al.* Polymorphisms of the human leukocyte antigen DRB1 and DQB1 genes and the natural history of human papillomavirus infection. *J Infect Dis* 2002; **186**: 164–72.
22 Mahmud SM, Robinson K, Richardson H *et al.* HLA polymorphisms and cervical human Papillomavirus infection in a cohort of Montreal University students. *J Infect Dis* 2007; **196**: 82–90.
23 Nakagawa S, Yoshikawa H, Yasugi T *et al.* Ubiquitous presence of E6 and E7 transcripts in human papillomavirus-positive cervical carcinomas regardless of its type. *J Med Virol* 2000; **62**: 251–8.
24 Coquillard G, Palao B, Patterson BK. Quantification of intracellular HPV E6/E7 mRNA expression increases the specificity and positive predictive value of cervical cancer screening compared to HPV DNA. *Gynecol Oncol* 2011; **120**: 89–93.
25 Yang YC, Chang TY, Lee YJ *et al.* HLA-DRB1 alleles and cervical squamous cell carcinoma: experimental study and meta-analysis. *Hum Immunol* 2006; **67**: 331–40.
26 Apple RJ, Erlich HA, Klitz W *et al.* HLA DR-DQ associations with cervical carcinoma show papillomavirus-type specificity. *Nat Genet* 1994; **6**: 157–62.
27 Cuzick J, Terry G, Ho L *et al.* Association between high-risk HPV types, HLA DRB1* and DQB1* alleles and cervical cancer in British women. *Br J Cancer* 2000; **82**: 1348–55.
28 Matsumoto K, Yasugi T, Nakagawa S *et al.* Human papillomavirus type 16 E6 variants and HLA class II alleles among Japanese women with cervical cancer. *Int J Cancer* 2003; **106**: 919–22.
29 Darragh TM, Colgan TJ, Cox JT *et al.* Members of LAST Project Work Groups. The lower anogenital squamous terminology standardization Project for HPV-Associated Lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. *Arch Pathol Lab Med* 2012; **136**: 1266–97.

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RESEARCH ARTICLE Open Access

HPV-16 impairs the subcellular distribution and levels of expression of protein phosphatase 1γ in cervical malignancy

Takayuki Seiki[1], Kazunori Nagasaka[1*], Christian Kranjec[2], Kei Kawana[1], Daichi Maeda[3], Hiroe Nakamura[1], Ayumi Taguchi[1], Yoko Matsumoto[1], Takahide Arimoto[1], Osamu Wada-Hiraike[1], Katsutoshi Oda[1], Shunsuke Nakagawa[4], Tetsu Yano[5], Masashi Fukayama[3], Lawrence Banks[2], Yutaka Osuga[1] and Tomoyuki Fujii[1]

Abstract

Background: The high risk Human Papillomavirus (HPV) E6 oncoproteins play an essential role in the development of cervical malignancy. Important cellular targets of E6 include p53 and the PDZ domain containing substrates such as hScrib and Dlg. We recently showed that hScrib activity was mediated in part through recruitment of protein phosphatase 1γ (PP1γ).

Methods: Expression patterns of hScrib and PP1γ were assessed by immunohistochemistry of HPV-16 positive cervical intraepithelial neoplasm (CIN), classified as CIN1 (n = 4), CIN2 (n = 8), CIN3 (n = 8), cervical carcinoma tissues (n = 11), and HPV-negative cervical tissues (n = 8), as well as by subfractionation assay of the HPV-16 positive cervical cancer cell lines, CaSki and SiHa. To explore the effects of the HPV-16 oncoproteins, we have performed siRNA knockdown of E6/E7 expression, and monitored the effects on the expression patterns of hScrib and PP1γ.

Results: We show that PP1γ levels in HPV-16 positive tumour cells are reduced in an E6/E7 dependent manner. Residual PP1γ in these cells is found mostly in the cytoplasm as opposed to the nucleus where it is predominantly found in normal cells. We have found a striking concordance with redistribution in the pattern of expression (9/11; 81.8%) and loss of PP1γ expression in HPV-16 positive cervical tumours (2/11; 18.2%). Furthermore, this loss of PP1γ expression and redistribution in the pattern of expression occurs progressively as the lesions develop (8/8; 100%).

Conclusion: Together, these results suggest that PP1γ may be a novel target of the HPV-16 oncoproteins and indicate that it might be a potential novel biomarker for HPV-16 induced malignancy.

Keywords: Cervical cancer, Immunohistochemistry, hScrib, Protein phosphatase 1, Proteasome degradation, Human papillomavirus 16

Background

Human Papillomaviruses (HPVs) are the aetiological agents of cervical cancer [1]. This is caused by infection with the high risk subset of HPV types, of which HPV-16 is the most important, being responsible for over 60% of global cervical cancer cases [2]. Cancer-causing HPVs encode two oncoproteins, E6 and E7, whose continued expression and activity is essential for maintaining the malignant phenotype, many years after the initial immortalising events [3,4]. Both viral oncoproteins function by perturbing the normal activity of a variety of different cellular control mechanisms. HPV E7 promotes cell cycle progression, in part through its association with members of the pocket protein family of tumour suppressors [5], whilst HPV E6 counteracts the pro-apoptotic effects of E7 through targeting the p53 tumour suppressor [6]. In both cases, the viral oncoproteins make efficient use of the cellular ubiquitin-proteasome machinery, with E7 targeting pRb through the cullin 2 ubiquitin ligase complex [7], whilst E6 uses the E6AP ubiquitin ligase to target p53 [8]. The effects of E6 and E7 are therefore cooperative, and this is reflected both in tissue culture systems, where they

* Correspondence: nagasakak-tky@umin.ac.jp
[1]Department of Obstetrics and Gynecology, Faculty of Medicine, The University of Tokyo, Tokyo 113-8655, Japan
Full list of author information is available at the end of the article

cooperate in the immortalisation of primary keratinocytes [9-11], and in animal models of tumourigenesis, where they cooperate in the induction of tumours in the skin and cervix [12,13].

Whilst targeting the pRb and p53 pathways is obviously very important for cervical tumourigenesis, it is also clear that E6 and E7 have a large number of other activities, many of which are also important for tumour development. In the case of high risk HPV E6 oncoproteins, an intriguing class of targets that appear to be important for HPV E6 induced malignancy are the PDZ (PSD/Dlg/ZO) domain containing substrates [14,15]. These are bound by E6 via a short stretch of amino acids within the extreme carboxy terminal region of the E6 oncoprotein. Most importantly, this PDZ binding motif (PBM) is only found in the high risk HPV E6 oncoproteins and is absent from the benign HPV E6 proteins [16,17]. Through this PBM, E6 can interact with a large number of cellular PDZ domain containing proteins, many of which are subject to E6-induced proteasomal degradation and E6-induced redistribution [16,18-21]. One of the most important of these targets is the cellular tumour suppressor hScrib. In Drosophila Scrib was originally identified as a potential tumour suppressor [22], and more recent studies in mammalian tissues also indicate tumour suppressive potential for hScrib. Loss of Scrib cooperates with c-Myc in the development of mammary carcinogenesis and Scrib also downregulates ERK signaling, with hScrib deregulation correlating with poor cancer prognosis [23-27]. In cervical tumourigenesis, hScrib patterns of expression are also perturbed as lesions develop, with hScrib being completely absent in many late stage tumours [28]. We recently found that hScrib could interact with PP1γ [29] a protein phosphatase that plays a critical role in controlling chromatin organization and also has an important role in the DNA damage response pathway [30,31] This suggested that PP1γ expression patterns in cervical tumourigenesis might likewise be perturbed. Therefore we initiated a series of studies to investigate the pattern of PP1γ expression in HPV16 positive cervical tumours and derived cell lines. We show that PP1γ is indeed subject to a striking alteration in both its levels of expression and localisation, both as lesions develop, and in the tumour derived cell lines. However this altered pattern of expression is independent of hScrib, is due directly to E6/E7 expression, and highlights PP1γ as potential novel biomarker of HPV induced neoplasia.

Methods

Cell lines and culture

HPV positive cervical cancer cell lines, CaSki, SiHa and HeLa plus HPV negative C33A (cervical cancer derived) and HaCaT (human keratinocytes) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2 [32]. The effect of proteasome inhibitor was determined 24 hours post-transfection after 3 hours of treatment with 10 μM MG132 (Calbiochem).

For plasmid transfection, 293 cells were transfected using TransIT-293 transfection reagent (Mirus Bio) and HaCaT cells were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, with pcDNA-HPV-16 E6. A plasmid expressing β-galactosidase was included in each transfection and pcDNA was used to equalize the input DNA.

Antibodies

The following commercial antibodies were used at the dilution indicated: anti-hScrib goat polyclonal antibody (Santa Cruz WB 1:1000, IHC 1:100), anti-PP1γ goat polyclonal antibody (Santa Cruz WB 1:1000), anti-PP1 Gamma/PPP1CC Antibody LS-B4960 IHC-plus (tm) rabbit polyclonal antibody (Lifespan bioscience, Inc. IHC 1:200), anti-PP1γ sheep polyclonal antibody (Abcam, WB 1:1000), anti-actin monoclonal antibody (Sigma, WB 1:5000), mouse monoclonal anti-p53 (DO-1) (Santa Cruz WB 1:500), anti-p84 mouse monoclonal antibody (Abcam, WB 1:1000), anti-E-Cadherin rabbit polyclonal antibody (Santa Cruz WB 1:500), anti-α-tubulin mouse monoclonal antibody (Abcam, WB 1:1000), mouse monoclonal anti-vimentin antibody (Santa Cruz WB 1:500).

siRNA transfection

The HPV-positive cervical cancer cells were seeded on 6 cm dishes and transfected using Lipofectamine 2000 (Invitrogen) with control siRNA against Luciferase (siLuc), or siRNA against HPV-16 and 18 E6 sequences (Dharmacon) described previously by Kranjec C et al., 2011. 72 hours post-transfection cells were harvested and total cell extracts or cell fractionated extracts were then analysed by western blotting. Alexa 568 labeled negative control siRNA (Qiagen) was used to measure transfection efficiency. The transfection efficiency was determined to be over 70% for each cell line.

Subcellular fractionation assays

Differential extraction of the cells to obtain cytoplasmic, membrane, cytoskeleton, and nuclear fractions was performed using the Calbiochem Proteo Extract Fractionation Kit according to the manufacturer's instructions. To inhibit phosphatase activity during the preparation of cell lysates, phosphatase inhibitors (1 mM Na_3VO_4, 1 mM β-Glycerophosphate, 2.5 mM Sodium Pyrophosphate, 1 mM Sodium Fluoride) were also included.