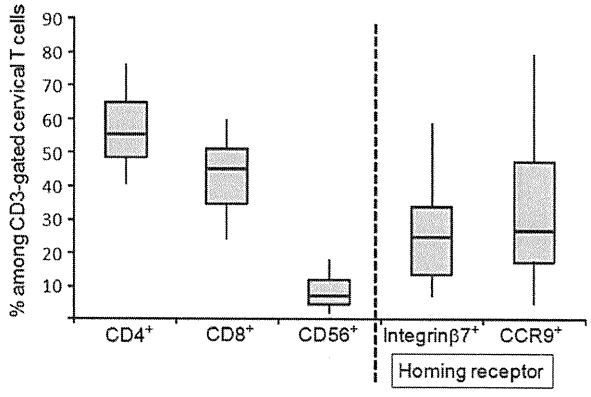

Fig. 2 Characterization of the cervical $CD3^+$ lymphocytes. CD3-gated cervical T cells consisted of $CD3^+$ $CD4^+$ T cells [median 54% (IQR: 49–65), $n = 28$], $CD3^+$ $CD8^+$ T cells [median 46% (IQR: 35–51), $n = 28$], and $CD3^+$ $CD56^+$ natural killer T cells [median 5.6% (IQR: 4.5–12), $n = 17$]. Twenty-four percentage (IQR: 13–34, $n = 43$) and 27% (IQR: 17–47, $n = 27$) of cervical T cells were integrin $\beta7^+$ and $CCR9^+$, respectively. Each median, IQR, and maximum/minimum range is indicated using horizontal lines, boxes, and vertical length lines, respectively.

(IQR: 49–65) of cervical T cells were $CD3^+$ and $CD4^+$, while a median of 46% (IQR: 35–51) expressed CD3 and CD8, demonstrating that $CD8^+$ T cells are relatively abundant among cervical T cells. The CD4/CD8 ratio of 1.15 in the cervix was clearly lower than the value of 2.0 found in peripheral blood. Among $CD3^+$ cells, a median of 5.6% (IQR: 4.5–12) were $CD56^+$ natural killer T (NKT) cells.

Those cervical $CD3^+$ T cells that were originally derived in the gut were defined by expression of the gut mucosa–specific cell-surface antigens integrin $\beta7^+$ and $CCR9^+$. A median of 24% (IQR: 13–34) of cervical T cells expressed integrin β7 and 27% (IQR: 17–47) expressed CCR9 (Fig. 2).

Notably, more than 90% (median: 99.1, IQR: 95.3–100) of the integrin $\beta7^+$ cells co-expressed the αE subunit (integrin $\alpha E\beta7^+$ cells; Fig. 3a). Integrin $\alpha4^+$ cells were rarely present among the integrin $\beta7^+$ cells (Fig. 3b). Approximately 40% (median: 40.1, IQR: 33.2–44.2) of cervical integrin $\beta7^+$ cells were integrin $\alpha E\beta7^+$ $CCR9^+$ double positive (Fig. 3c).

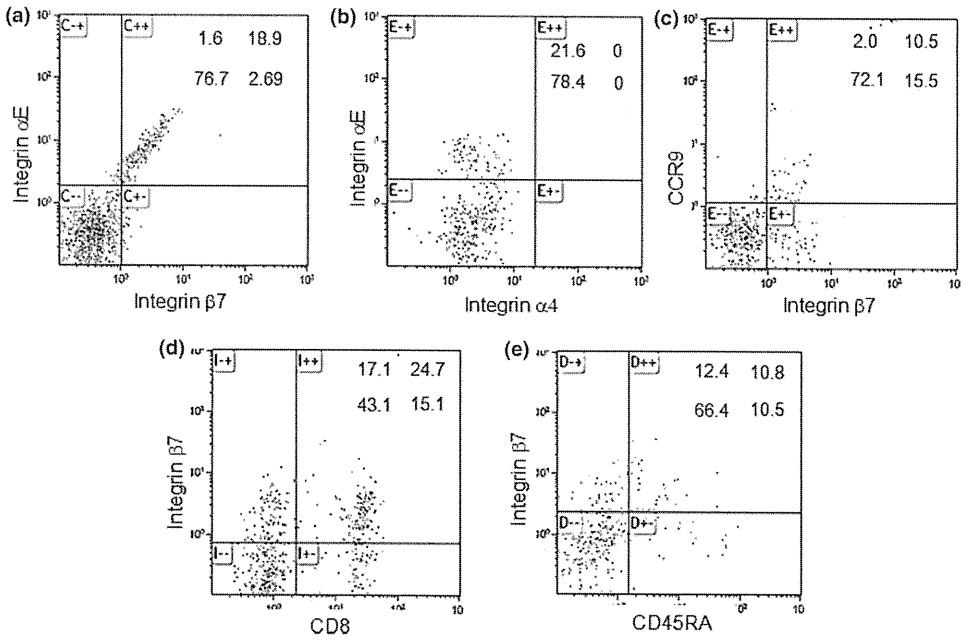

Fig. 3 Characterization of CD8, CD45RA, and homing receptors specific for gut-derived mucosal T cells among $CD3^+$ cervical T cells. Representative flow cytometry analyses of CD3-gated cervical T cells. (a) More than 90% of integrin $\beta7^+$ cervical T cells were integrin αE^+ intraepithelial lymphocyte. (b) Integrin $\alpha4^+$ LPL were negligible in our cervical samples. (c) Among integrin $\beta7^+$ cells, approximately 40% were $CCR9^+$. (d) Forty-two percentage of total cervical T cells and 53% of integrin $\beta7^+$ T cells were $CD8^+$. (e) About half of the integrin $\beta7^+$ T cells were CD45RA-negative memory cells.

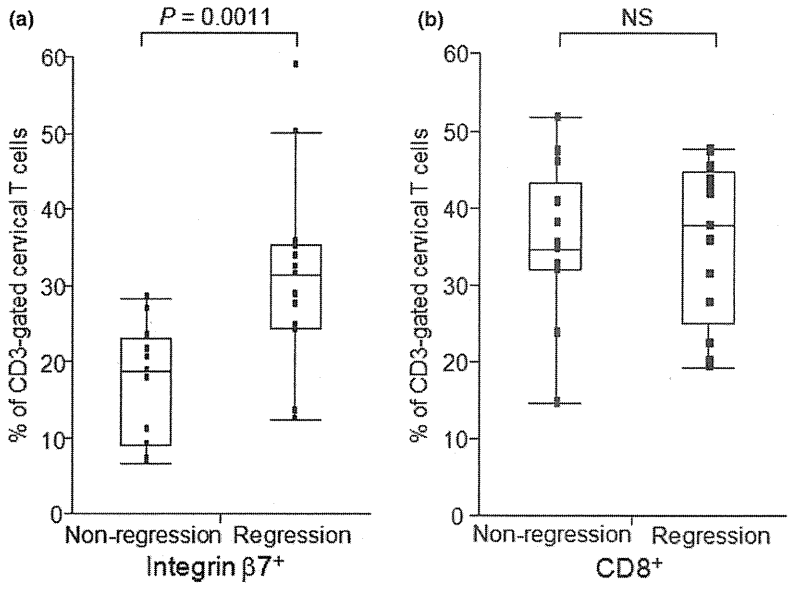

Fig. 4 Association between gut-derived cervical intraepithelial lymphocyte and cervical intraepithelial neoplasia (CIN) regression. Populations of integrin $\beta7^+$ (a) and $CD8^+$ (b) cells among $CD3^+$ cervical T cells were compared between CIN regression (n = 13) and non-regression (n = 13) groups, paired according to follow-up duration. A P-value ≤0.05 was considered significant using Wilcoxon rank test comparisons.

$CD8^+$ memory T cells are essential for adaptive cytotoxic immune responses to CIN.[21,22] Among our patients with CIN, the proportion of integrin $\beta7^+$ cervical T cells that expressed CD8 [median 53% (IQR: 28–69)] was greater than that for total cervical T cells (Fig. 3d). Approximately half [median 43% (IQR: 31–57)] of integrin $\beta7^+$ T cells were CD45RO memory T cells, while the other half were CD45RA effector T cells (Fig. 3e).

Association of Gut-derived Cervical IEL with CIN Course

Integrin β7 is a more ubiquitous homing receptor in mucosal lymphocytes rather than integrin αE or α4. To determine whether there was an association between the presence of gut-derived cervical IEL and spontaneous regression of CIN, comparisons were made between populations of integrin $\beta7^+$ $CD3^+$ and $CD8^+$ $CD3^+$ cervical T cells in CIN regressors (n = 13) and non-regressors (n = 13), paired according to their duration of follow-up. No significant differences were seen in the detection rates of high-risk HPV (69 versus 77%, P = 0.50), the squamous intraepithelial lesion (SIL) grade (high-grade SIL: 54 versus 54%, P = 0.65), and the median ages (34 years old versus 35) of patients in the regression and non-regression groups. The percentage of integrin $\beta7^+$ cervical T cells varied from 6 to 57% among the 26 study subjects. Among regressors, integrin $\beta7^+$ cervical T cells comprised a median of 31.6% (IQR: 24.5–35.5) of $CD3^+$ cervical T cells; the rate among non-regressors was 18.8% (IQR: 9.2–23.3), P = 0.0011 (Fig. 4a). In contrast, there was no difference in populations of $CD8^+$ $CD3^+$ cervical T cells between CIN regressors and non-regressors (Fig. 4b). The proportion of $CCR9^+$ and $CD45RA^+$ $CD3^+$ cervical T cells was likewise similar in the two groups (data not shown).

Discussion

Human papillomavirus preferentially infects, and CIN develops in the human cervical epithelium. It is clear that HPV antigens are recognized by the systemic cell-mediated immune system, but remains unclear whether systemic cellular immune responses predict the regression of CIN.[16,17] Local mucosal immune responses in the cervix are likely to be important in immunological clearance of CIN lesions. Integrin $\alpha4\beta7$ is essential for recruiting activated mucosal lymphocytes from the circulation into local LP in a manner entirely dependent on interaction between lymphocyte integrin $\alpha4\beta7$ and the MAdCAM-1 that is constitutively expressed on LP post-capillary venules.[23] In contrast, integrin αE (CD103) β7 is expressed by only 2% of circulating blood lymphocytes, but more than 90% of IEL and a minority of lamina propria lymphocyte (LPL); its ligand is E-cadherin expressed on the epithelial cells.[24] Activated integrin $\alpha4\beta7^+$ T cells differentiate within the

LP into integrin $\alpha E\beta7^+$ T cells upon exposure to TGF-β locally secreted by epithelial cells.[5] Binding of integrin $\alpha E\beta7$ to E-cadherin provokes retention of the activated IEL within the epithelium. Recognition of target epithelial cells by IELs is important in the initiation of cytolytic effector function by activated IELs and modulation of adaptive immune responses to control potentially destructive epithelial immunity. Adhesion of integrin $\alpha E\beta7^+$ IEL to epithelial E-cadherin is promoted by CCL25–CCR9 interactions.[4] This suggests that, when compared to integrin $\alpha4\beta7^+$ LPL, integrin $\alpha E\beta7^+$ IELs may be more directly linked to essential adaptive immune responses to target epithelial cells at local effector sites.

Several studies have reported that integrin $\alpha4\beta7$ is expressed on gut-derived mucosal lymphocytes within the cervix.[9,11] However, our data indicate that more than 90% of integrin $\beta7^+$ T cells were positive for integrin αE and few express α4. Pudney et al.[10] have shown using immunohistochemistry that integrin $\alpha E\beta7^+$ lymphocytes are primarily located in the epithelium of the ectocervix and often occur as focal accumulations in the LP of the transformation zone. Our brushing methodology enables us to preferentially collect cervical mucosal lymphocytes from the epithelium and occasionally from the LP. Others who have recently reported that nearly all cervical tissue T cells are integrin $\alpha4\beta7^{+9}$ used cervical tissue specimens and equally valuable methodologies that would be expected to isolate cells from deeper within the cervical tissue, possibly favoring isolation of LPL over cells tightly adhered to the epithelium.

Our cervical samples were contaminated by numerous granulocytes, a finding supported by several previous studies using cervical mucosa unlike peripheral blood samples.[10,11] Granulocyte contamination variability was likely the result of differing levels of cervical inflammation among patients. Although the number of lymphocytes among $CD45^+$ cervical leukocytes varied from 10 to 30%, the absolute number of cervical lymphocytes present in a sample appeared to be relatively constant and independent of patient source. The efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin β7 and CCR9. We showed that integrin β7 and CCR9 did not always co-express. This agrees with reports showing that expression of the mucosal homing receptors, integrin β7 and CCR9, is not always linked, but instead depends on lymphocyte differentiation and the location of the effector sites infiltrated by these cells.[25,26]

Expression of MAdCAM-1 is essential for trafficking of integrin $\alpha4\beta7^+$ lymphocytes into the LP, while the expression of E-cadherin on the epithelium is essential for the retention of integrin $\alpha E\beta7^+$ lymphocytes. Inflammation of the mucosa enhances MAdCAM-1 expression on the endothelial cells of post-capillary venules in the genital tract,[8] and inflammatory changes are often observed in CIN when compared with normal cervical mucosa.[27,28] Trimble et al.[9] reported that MAdCAM-1 expression correlates with non-specific $CD8^+$ LPL infiltration of the LP and CIN regression. In our sampled IELs, there was no association between $CD8^+$ cells and CIN regression. Studies have also demonstrated that oncoproteins from high-risk HPV subtypes downregulate E-cadherin expression in CIN lesions and that this downregulation is closely associated with disease progression.[29–31] E-cadherin plays an important role in the maintenance of normal adhesion in epithelial sites and its loss is associated with poor prognosis for many tumors other than CIN.[32] The downregulation of E-cadherin may interfere with the retention of integrin $\alpha E\beta7^+$ T cells in CIN lesions, and our results suggest that IEL retention varies among patients with CIN. We have shown that populations of integrin $\alpha E\beta7^+$ IEL in CIN lesions vary markedly among patients and that higher IEL numbers are associated with spontaneous regression of CIN. Although HPV-specific cytotoxic T lymphocyte activity was not investigated here, the accumulation of integrin $\alpha E\beta7^+$ IEL in CIN lesions and their association with CIN regression suggests these cells, rather than non-specific $CD8^+$ T cells, may have important local effector functions in the cervical epithelium. In the present study, the adaptive immune system was focused, but the innate immune responses play equally important roles in controlling HPV infection. Daud et al.[33] has recently reported the mechanism of interference with innate immune system by HPV16, dampened toll-like receptor expression, which results in the viral persistence. The interaction of innate with adaptive immunity at the local mucosa should be investigated.

In summary, our report is the first to specifically phenotype cervical IEL in CIN lesions. Our results indicate that the presence of elevated numbers of gut-derived integrin $\alpha E\beta7^+$ IELs in specimens gathered from patients with CIN using a cervical cytobrush may represent a possible biomarker for CIN regression. Sampling of cervical IEL using this methodology is relatively non-invasive and techni-

cally easier than the isolation of cervical LPL from tissue biopsies. Future investigations using our sampling methods will focus on HPV-specific cell-mediated immune responses by cervical IELs isolated from patients with CIN. These and related investigations should improve our understanding of cervical mucosal immunity and hasten the development of a therapeutic HPV vaccine.

Acknowledgements

This work was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan for the Third-Term Comprehensive 10-Year Strategy for Cancer Control, by a cancer research grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant from the Okinawa New Industry Creation Project.

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Med Oncol
DOI 10.1007/s12032-011-9893-0

CASE REPORT

Interstitial pneumonitis induced by pegylated liposomal doxorubicin in a patient with recurrent ovarian cancer

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Received: 24 February 2011 / Accepted: 28 February 2011

Abstract Interstitial pneumonitis after treatment with pegylated liposomal doxorubicin (PLD) has been rarely reported. We describe herein a case of interstitial pneumonitis in a 49-year-old woman with relapsed ovarian carcinoma treated with PLD. Twenty-five days after the second administration of PLD, she presented with fever and dry cough, and chest CT scans revealed bilateral interstitial infiltrates and ground-glass opacities. She was diagnosed to have interstitial pneumonitis induced by PLD. Steroid therapy improved her symptoms.

Keywords Interstitial pneumonitis · Pegylated liposomal doxorubicin · Drug induced · Japanese · Ovarian cancer

Introduction

Pegylated liposomal doxorubicin (PLD) is an active drug in recurrent ovarian cancer as demonstrated in trials in the second-line chemotherapy [1–3]. It has been designed to enhance the efficacy and to reduce the toxicities of doxorubicin such as cardiotoxicity, hematologic toxicity, and alopecia by using a unique delivery system: a polyethylene glycol coat [4, 5]. Whereas hand-foot syndrome and planter palmar erythema are widely recognized as adverse effects of PLD, few cases of interstitial pneumonitis after treatment with PLD have been reported. Here, we describe a case of interstitial pneumonitis induced by PLD.

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Case report

A 48-year-old woman (gravida 4, para 3) with recurrent ovarian cancer was started on third-line chemotherapy with PLD (50 mg/m^2/4 weeks). She was initially diagnosed in February 2009 and underwent complete debulking surgery for a stage IIIC serous ovarian adenocarcinoma. Postoperatively, she received adjuvant chemotherapy with six cycles of paclitaxel (175 mg/m^2) and carboplatin (AUC 6). Four months later, because of peritoneum dissemination and elevation of CA125, she was treated with weekly CPT-11 (95 mg/m^2/week) with progressive disease after four cycles. In April 2010, PLD was given under her excellent performance status.

Twenty-three days after the first administration of PLD, she developed a fever from which she recovered without any treatment. However, 25 days after the second administration of PLD, she presented to our hospital with fever, chill, dry cough, and dyspnea (grade 3 according to Common terminology criteria for adverse events, version 4.0). Physical examination was remarkable for bilateral fine crackles at the lung bases. A chest X-ray and chest CT scans revealed bilateral interstitial infiltrates and ground-glass opacities, though chest CT scans performed before PLD therapy showed clear lung field (Fig. 1a, b). Oxygen saturation by pulse oximetry was 89% on room air and arterial blood gas analysis showed hypoxia (FiO_2 0.32, PaO_2 90.5 mmHg, alveolar-arterial oxygen gradient 94.9 mmHg). Laboratory analysis revealed white blood cells of 2,500/μl with 78% neutrophils, lactate dehydrogenase of 347 IU/l, C-reactive protein of 14.32 mg/dl, and Krebs von den Lungen-6 (KL-6) of 227 U/ml.

Her clinical course and laboratory data indicated that she has interstitial pneumonitis probably induced by PLD. She had not received granulocyte colony-stimulating

Published online: 10 March 2011

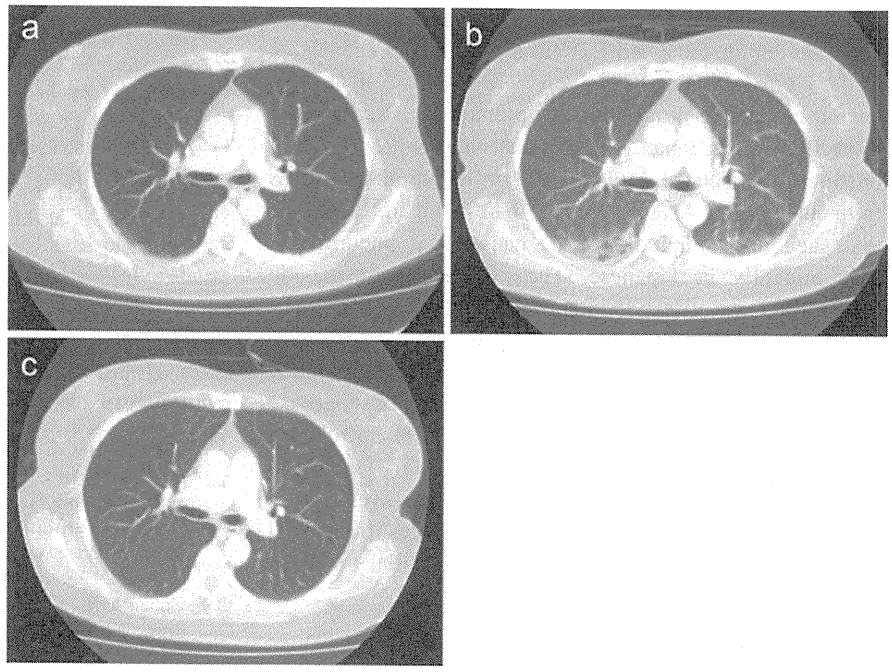

Fig. 1 a Chest computed tomography (CT) scan before PLD therapy showed clear lung field. b Twenty-six days after second administration of PLD, CT revealed bilateral interstitial infiltrates and ground-glass opacities. c Two months after steroid therapy, CT showed significant improvement

factor, which could induce interstitial pneumonitis. In addition to PLD, she received ascorbic acid, pyridoxal phosphate hydrate, rebamipide, and brotizolam. As they were all unlikely to induce interstitial pneumonitis, administration of these drugs except PLD was continued. The patient was treated with intravenous methylprednisolone 500 mg/day for 3 days. Azithromycin 1,000 mg per os and intravenous cefepime 4 g/day were administrated until all examinations of infection proved to be negative, including blood culture, β-D-glucan, influenza antigen detection, urinary pneumococcal antigen test, Chlamydia IgA/IgG, candida antibody assays, and galactomannan antigen of aspergillosis.

After the steroid pulse therapy, symptoms resolved promptly and lung function tests improved remarkably. Two months after the diagnosis of interstitial pneumonitis, a chest CT scan showed significant improvement (Fig. 1c). PLD was discontinued and her chemotherapy regimen was changed to docetaxel (70 mg/m^2). She has not shown any respiratory symptoms after cessation of PLD. Currently, she is alive with disease 24 months after the surgery and undergoing fifth-line chemotherapy.

Discussion

Pegylated liposomal doxorubicin is a reformulated version of doxorubicin, which takes the active agent doxorubicin and places it into a phospholipid bilayer called a liposome and another outer layer of methoxypolyethylene glycol. This coating allows PLD to evade detection and destruction by the immune system and to remain longer in the blood circulation.

PLD has a different toxicity profile compared with free doxorubicin. Though cumulative cardiac toxicities are unique to free doxorubicin, cardiac toxicities associated with PLD are rarely reported. Toxicities relatively unique to PLD are hand-foot syndrome or plantar palmar erythema (PPE), which are rarely reported with free doxorubicin.

It is reported that lung toxicity induced by doxorubicin is rare. Several cases of interstitial pneumonitis associated with doxorubicin or PLD have been described [6, 7]. It was unclear whether the lung toxicities were directly attributable to doxorubicin in published case reports, because all patients were concurrently receiving other agents, mostly antineoplastic drugs, which were also implicated in causing lung toxicity.

In our case, though the symptoms were initially severe, discontinuation of PLD and steroid therapy immediately resolved them. Serum KL-6 levels have been reported to correlate with grade of interstitial lung disease [8]. Normal serum KL-6 level in this case might associate with her excellent clinical course.

Two possible mechanisms of drug-induced interstitial pneumonitis have been described, one of which is the direct toxicity of the drug to the pulmonary organ and the other is immunological mechanism, although the etiology of PLD-induced interstitial pneumonitis is unclear.

Drug-induced pulmonary toxicity in Japan got a great deal of attention because of pulmonary toxicity induced by molecular-targeted chemotherapeutic drugs, gefitinib and an antirheumatic drug, leflunomide. It is reported that the

rates of interstitial lung disease associated with gefitinib and leflunomide are 2 and 1.1% in Japan and 0.3 and 0.02% in the United States, respectively. These data indicate that chemotherapeutic-drug-induced pulmonary toxicity is more frequent in Japan than in other nations [9, 10]. Fatal pneumonitis induced by gefitinib or leflunomide is less frequent in other Asian countries than in Japan. It may be that such drugs including PLD cause fatal pneumonitis predominantly in Japanese. The differences of genetic background or lifestyle between Japanese and non-Japanese might be involved in this event.

Drug-induced interstitial pneumonitis should be taken into consideration in the differential diagnosis of otherwise unexplained ground-glass lung lesions. Pulmonary toxicity induced by PLD is rare, but awareness of this toxicity is important, since it could be lethal. Additional investigation is required to elucidate how PLD induces interstitial pneumonitis or whether PLD-induced interstitial pneumonitis is more frequent in Japanese.

Conflict of interest No author has any conflict of interest.

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Med Oncol
DOI 10.1007/s12032-011-9878-z

LETTER TO THE EDITOR

Second-line chemotherapy with docetaxel and carboplatin in paclitaxel and platinum-pretreated ovarian, fallopian tube, and peritoneal cancer

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Abstract We retrospectively evaluated the efficacy and toxicity of docetaxel and carboplatin in patients with platinum and paclitaxel-pretreated recurrent ovarian, fallopian tube, and peritoneal cancer. Forty-two women (38 with ovarian cancer, 1 with fallopian tube cancer, 3 with peritoneal cancer) whose cancer had progressed within 12 months of their last treatment with both a platinum agent and paclitaxel were treated with docetaxel (70 mg/m^2, day 1) and carboplatin (area under the curve of 4–6, day 1). Thirty-four patients had measurable disease. The objective response rate was 23% within 0–6 months of the progression-free interval, 50% within 6–12 months, and 32% (11 of 34 patients) for both groups. The median time to tumor progression was 28, 49, 34 weeks, and the median overall survival time was 94, 224, 111 weeks, respectively. The most common toxicity was grade 3/4 neutropenia (98% of patients), with 15 episodes (8.4% of courses) of neutropenic fever. The main nonhematologic toxicity was hypersensitivity; 7 patients (17%) required discontinuation of the therapy. The results of our study indicate that the combination of docetaxel and carboplatin is effective against recurrent ovarian, fallopian tube, and peritoneal cancer with progression-free interval of 6–12 months from previous treatment by paclitaxel and platinum. On the other hand, single-agent chemotherapy would be better than this regimen considering its low response rate and severe hematological toxicity for patients with progression-free interval less than 6 months.

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Published online: 06 March 2011

Keywords Docetaxel · Carboplatin · Chemotherapy · Early progression · Recurrent ovarian cancer

The standard regimen as second-line chemotherapy in recurrent ovarian cancer has not been established, especially in the patients with a short progression-free interval from the previous treatment. Docetaxel is an active drug as second-line chemotherapy for recurrent ovarian cancer as well as pegylated liposomal doxorubicin, irinotecan, topotecan, gemcitabine, and etoposide [1].

The purpose of this study was to evaluate activity and toxicity of the combination of docetaxel and carboplatin retrospectively in patients with paclitaxel and platinum resistant (progression-free interval less than 6 months) and partially resistant (progression-free interval of 6–12 months) ovarian, fallopian tube, and peritoneal cancers. Forty-two women (38 with ovarian cancer, 1 with fallopian tube cancer, 3 with peritoneal cancer) whose cancer had progressed within 12 months of their last treatment with both a platinum agent and paclitaxel were treated with docetaxel (70 mg/m^2, day 1) and carboplatin (area under the curve of 4–6, day 1). Thirty-four (81%) patients had measurable disease. Twenty-six (62%) patients had experienced progression of disease within less than 6 months of their last treatment, whereas 16 patients (38%) within 6–12 months. The median number of courses of treatment per patient was 4.5 (range: 1–8 courses). The median follow-up period was 107 weeks (range: 9–373 weeks). The objective response rate was 23% within 0–6 months of the progression-free interval, 50% within 6–12 months, and 32% (11 of 34 patients) for both groups. The median time to tumor progression was 28, 49, and 34 weeks, and the median overall survival time was 94, 224, and 111 weeks, respectively. The most common toxicity was grade 3/4 neutropenia (98% of patients), with 15 episodes

(8.4% of courses) of neutropenic fever. The main nonhematologic toxicity was hypersensitivity; 7 patients (17%) required discontinuation of the therapy. On the other hand, grade 2/3 neuropathy was observed only in two (4.8%) patients.

Several chemotherapeutic agents such as pegylated liposomal doxorubicin, topotecan, irinotecan, gemcitabine, and etoposide have been used in the treatment of platinum-resistant disease with response rates in the range 10–15% [2–5]. The results from our study about overall response rate are in line with other chemotherapeutic agents. Notably, our data about median time to tumor progression and overall survival are longer than the previously reported data of other regimens.

The results of our study indicate that the combination of docetaxel and carboplatin is effective against recurrent ovarian, fallopian tube, and peritoneal cancer with progression-free interval of 6–12 months from previous treatment by paclitaxel and platinum. On the other hand, single-agent chemotherapy would be better than this regimen considering its low response rate and severe hematological toxicity for patients with progression-free interval less than 6 months. However, chemotherapy with docetaxel and carboplatin may improve time to tumor progression and overall survival time in these cases; this regimen can be an alternative in patients whose hematological toxicity is relatively weak at their previous treatment.

Conflict of interest None.

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Molecular Analyses of TEM Genes and Their Corresponding Penicillinase-Producing *Neisseria gonorrhoeae* Isolates in Bangkok, Thailand

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***Neisseria gonorrhoeae* is a major public health problem globally, especially because the bacterium has developed resistance to most antimicrobials introduced for first-line treatment of gonorrhea. In the present study, 96 *N. gonorrhoeae* isolates with high-level resistance to penicillin from 121 clinical isolates in Thailand were examined to investigate changes related to their plasmid-mediated penicillin resistance and their molecular epidemiological relationships. A β-lactamase (TEM) gene variant, $bla_{TEM-135}$, that may be a precursor in the transitional stage of a traditional bla_{TEM-1} gene into an extended-spectrum β-lactamase (ESBL), possibly causing high resistance to all extended-spectrum cephalosporins in *N. gonorrhoeae*, was identified. Clonal analysis using multilocus sequence typing (MLST) and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) revealed the existence of a sexual network among patients from Japan and Thailand. Molecular analysis of the $bla_{TEM-135}$ gene showed that the emergence of this allele might not be a rare genetic event and that the allele has evolved in different plasmid backgrounds, which results possibly indicate that it is selected due to antimicrobial pressure. The presence of the $bla_{TEM-135}$ allele in the penicillinase-producing *N. gonorrhoeae* population may call for monitoring for the possible emergence of ESBL-producing *N. gonorrhoeae* in the future. This study identified a bla_{TEM} variant ($bla_{TEM-135}$) that is a possible intermediate precursor for an ESBL, which warrants international awareness.**

Neisseria gonorrhoeae is the causative agent of gonorrhea, which is the second most prevalent bacterial sexually transmitted infection globally. During recent decades, *N. gonorrhoeae* has rapidly developed resistance to most classes of antimicrobials used for treatment of gonorrhea (4, 6, 17, 18, 20). Penicillinase-producing *N. gonorrhoeae* (PPNG), with plasmid-mediated high-level resistance to penicillin, was first reported in 1976 (1, 14) and has since been disseminated worldwide (2). The first gonococcal strain with high-level clinical resistance to ceftriaxone, which is the last remaining option for first-line gonorrhea treatment, was recently found in Japan and completely characterized (9, 11). However, the resistance to ceftriaxone was chromosomally mediated, and no extended-spectrum β-lactamase (ESBL) has yet been identified in *N. gonorrhoeae*. If an ESBL did emerge in *N. gonorrhoeae* and spread internationally, gonorrhea would become an extremely serious public health problem.

PPNG strains are rare in Japan, but these strains have remained highly prevalent in several other countries in Asia (19) and worldwide (20). Penicillin is still also used as the first-line drug in, e.g., some Pacific island countries and the northern part of Australia, because of maintained efficacy in the settings and its low cost.

Although the β-lactamase (TEM) gene of authentic PPNG is the bla_{TEM-1} allele, a recently isolated PPNG in Thailand possessed the $bla_{TEM-135}$ allele, which differs from the bla_{TEM-1} allele with one single nucleotide polymorphism (SNP) at position 539, resulting in a single amino acid substitution, M182T (16). However, the prevalence and characteristics of TEM-135 strains worldwide are unknown and seem critical to study, especially in countries where PPNG strains are highly prevalent. Furthermore, the knowledge regarding the genetic relationships of PPNG strains, their TEM genes, and plasmids carrying β-lactamase is highly limited.

Therefore, in the present study, PPNG isolates cultured from 2005 to 2007 in Thailand, which has a relatively high prevalence of PPNG, were investigated. To detect $bla_{TEM-135}$ in the PPNG strains, a simple and rapid mismatch amplification mutation assay (MAMA) PCR method (3) was developed and successfully used. To reveal the population structure of the PPNG isolates, molecular epidemiological typing by means of multilocus sequence typing (MLST) (5), *porB* gene sequencing, and *N. gonorrhoeae* multiantigen sequence typing NG-MAST (7) were used to compare the detected TEM-135 strains with the TEM-1 strains.

MATERIALS AND METHODS

Bacterial isolates. *N. gonorrhoeae* isolates were collected from Siriraj Hospital, Bangkok, Thailand. Among 121 isolates collected during 2005 to 2007, based on resistance to penicillin and a positive nitrocefin test, a total of 96 PPNG isolates were detected and analyzed (see the supplemental material). These isolates were systematically collected in a previous research project (16).

Received 2 September 2011 Returned for modification 15 October 2011
Accepted 27 November 2011
Published ahead of print 5 December 2011
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Supplemental material for this article may be found at http://aac.asm.org/.

doi:10.1128/AAC.05665-11

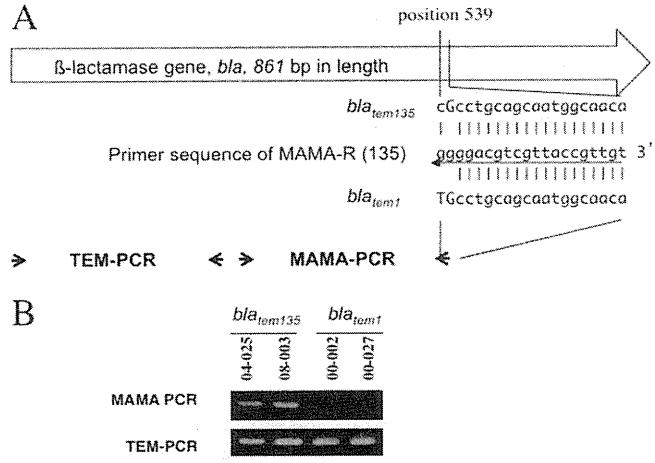

FIG 1 MAMA-PCR for $bla_{TEM-135}$ detection. (A) The TEM PCR primer set (TEM-F and TEM-R), which can amplify a 231-bp amplicon from bla_{TEM-1} and $bla_{TEM-135}$, and the MAMA-PCR primer set, specific for $bla_{TEM-135}$ (MAMA-F and MAMA-R), are shown schematically with arrows. The sequence of primer MAMA-R (middle) and the corresponding regions from bla_{tem135} (top) and bla_{tem1} (bottom) are also shown. (B) The PCR results for the Japanese penicillinase-producing *N. gonorrhoeae* (PPNG) TEM-135 and PPNG TEM-1 isolates, which were used as controls in all PCRs, are presented.

DNA isolation. To obtain genomic DNA, isolates were suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and boiled for 10 min. After removing cell debris by centrifugation, the supernatant was used directly as template DNA in the PCR.

PCR identification of bla_{TEM} gene. The MAMA-PCR to detect sequence polymorphism between the bla_{TEM-1} and $bla_{TEM-135}$ alleles focused on nucleotide position 539 of the bla_{TEM} gene (Fig. 1A). A conserved forward primer (MAMA-F, 5′-GCATCTTACGGATGGCATGA C-3′) and a $bla_{TEM-135}$ allele-specific polymorphism detection primer (MAMA-R, 5′-TGTTGCCATTGCTGCAGG**G**G-3′) were designed (Table 1). The $bla_{TEM-135}$ allele-specific primer carries a specific nucleotide, G (bold and underlined), at the 3′ end. Furthermore, to enhance the 3′ end mismatch effect, an additional nucleotide alteration of G, rather than C (bold), at the second nucleotide from the 3′ end of the primer was introduced. Thus, the $bla_{TEM-135}$ allele-specific primer contained two mismatched bases at the 3′ end relative to the sequence of bla_{TEM-1} (Fig. 1A). In brief, the 10-μl-volume PCR master mix contained diluted template DNA, 0.8 μl of 2.5 mM deoxynucleoside triphosphate (dNTP) mixture (final concentration, 200 μM each), 0.25 μl each of 10 μM MAMA-F and MAMA-R primers (final concentration, 250 nM each), and 0.25 units of the DNA polymerase Takara Ex *Taq* (Takara Bio Co., Kyoto, Japan). The parameters of the PCRs were as follows: incubation for 2 min at 96°C followed by 25 cycles of 10 s at 96°C, 10 s at 56°C, and 30 s at 72°C and then final extension for 2 min at 72°C. The previously described *N. gonorrhoeae* strains NGON 00-002 and NGON 00-027 (containing bla_{TEM-1}) and NGON 04-025 and NGON 08-003 (containing $bla_{TEM-135}$) (10) were used as controls in all PCRs (Fig. 1). The universal TEM PCR was done as described above except that the PCR master mix contained TEM-F and TEM-R primers (Table 1). To confirm TEM alleles, we sequenced PCR-amplified products of the whole bla_{TEM} coding region using the primer set bla-F and bla-R as described previously (10).

Molecular epidemiological characterization. Molecular epidemiological characterization by means of MLST (5), *porB* gene sequencing, and NG-MAST was performed as described previously (7). The type of plasmid carrying the β-lactamase (TEM) gene was determined by a multiplex PCR method developed by Palmer et al. (12). Neighbor-joining trees with *por* and *tbpB* nucleotide sequences were generated by using MEGA4.

Drawing of minimum spanning tree. Based on the MLST data, a minimum spanning tree was generated by using BioNumerics (version 5.1; Applied Math), using the categorical coefficient of similarity and the priority rule of the highest number of single-locus variants as parameters. No hypothetical sequence or reported sequences other than those identified in the present study were included in the calculation.

RESULTS AND DISCUSSION

Development and use of the MAMA-PCR for detection of $bla_{TEM-135}$. To differentiate the $bla_{TEM-135}$ allele from the bla_{TEM-1} allele in PPNG strains, by detection of the SNP at position 539, a MAMA-PCR (detecting only $bla_{TEM-135}$) was successfully developed and was used together with a TEM PCR (detecting both $bla_{TEM-135}$ and bla_{TEM-1}) (Fig. 1).

Nine of the 96 PPNG isolates from Thailand were positive in both the MAMA-PCR and TEM PCR, suggesting that these isolates possessed the $bla_{TEM-135}$ allele. Sequencing analysis of the full-length PCR products from the *bla* gene confirmed that these nine isolates (9.4%) indeed contained the $bla_{TEM-135}$ allele, and the remaining 87 isolates (90.6%) possessed bla_{TEM-1}.

Genetic relationships of PPNG TEM-1 and PPNG TEM-135 isolates. In order to examine the genetic relationships of PPNG isolates containing TEM-1 and TEM-135, MLST was carried out. Twenty-three MLST STs were identified among the 96 PPNG isolates, 17 STs among the TEM-1 isolates and 6 among the TEM-135 isolates. Among the 17 MLST STs identified among the TEM-1 isolates, ST1588 was the most prevalent (55 out of the 87 TEM-1 isolates, 63.2%) (Table 2). A minimum spanning tree analysis showed that most of the other STs in TEM-1 isolates were closely related to ST1588, with few exceptions (Fig. 2). Accordingly, 83 out of the 87 (95.4%) TEM-1 isolates belonged to a large cluster comprising 15 STs and centered around ST1588 (cluster A) (Fig. 2 and Table 2). The remaining four TEM-1 isolates were assigned ST8782 ($n = 2$) and ST8775 ($n = 2$), which formed an additional smaller cluster (cluster B) (Fig. 2 and Table 2).

Six different MLST STs were found in the nine TEM-135 isolates (Fig. 2 and Table 2). ST8778 was the most common ($n = 4$, 44.4%), and the other five STs were singletons. All these TEM-135 isolates, with the exception of the singleton ST7822 (isolate Thai_026) that was placed in the TEM-1 cluster A, belonged to the same separate cluster (cluster C) (Fig. 2 and Table 2). Taken together, Thailand PPNG TEM-1 and PPNG TEM-135 strains seem to belong to distinct clonal groupings with different genetic backgrounds, and also, TEM-135 strains have emerged from multiple independent origins.

Plasmid typing. Plasmid typing has been used as another classification method for PPNG surveillance. We also performed plasmid typing and investigated relationships with the results of MLST and the specific alleles of the *bla* genes bla_{TEM-1} and $bla_{TEM-135}$.

As shown in Table 2, the Africa-type β-lactamase plasmid was the predominant type (79 of 96 isolates, 82.3%) in the isolates

TABLE 1 Primers used in the MAMA-PCR for detection of $bla_{TEM-135}$ and the TEM-PCR for detection of both bla_{TEM-1} and $bla_{TEM-135}$

Primer	Primer sequence (5′ to 3′)	Position
MAMA-F	GCATCTTACGGATGGCATGAC	327-347
MAMA-R[a]	TGTTGCCATTGCTGCAGGGG	558-539
TEM-F	GTCGCCCTTATTCCCTTTTTTG	22-43
TEM-R	TAGTGTATGCGGCGACCGAG	284-268

[a] Binds only $bla_{TEM-135}$.

TABLE 2 MLST sequence type and plasmid type of PPNG isolates cultured in Thailand in 2005 to 2007[a]

No. of isolates	MLST		No. of isolates with plasmid type[c]:		
	ST	Cluster[b]	Africa	Asia	Toronto/Rio
55	1588	A	53	1	1
5	8780	A	5		
4	1903	A	4		
4	8774	A	4		
2	1584	A	2		
2	1921	A	2		
2	8779	A	2		
1	7827	A	1		
1	8145	A	1		
1	8776	A	1		
1	8783	A	1		
1	8777	A	1		
2	7823	A		2	
1	1600	A		1	
1	7822	A		1 (1)[d]	
1	8781	A		1	
2	8775	B		2	
2	8782	B	2		
4	8778	C			4 (4)
1	1582	C			1 (1)
1	8136	C			1 (1)
1	8143	C			1 (1)
1	8784	C			1 (1)
96		TOTAL	79	8 (1)	9 (8)

[a] MLST, multilocus sequencing typing (5); PPNG, penicillinase-producing *Neisseria gonorrhoeae*.
[b] Clusters were defined by the minimum spanning tree in Fig. 2.
[c] Plasmid typing was determined by a multiplex PCR (12).
[d] The number of PPNG TEM-135 isolates is shown in parentheses.

analyzed in the present study. Asia- and Toronto/Rio-type β-lactamase plasmids were found in only eight and nine isolates, respectively. Recently, a new type of the β-lactamase plasmid (Johannesburg plasmid) was reported by Muller et al. (8). If the Johannesburg-type plasmid had existed in our isolates, it would have generated a 450-bp amplicon with the BL1 and BL3 primers in our multiplex PCR system. However, we did not find any isolates containing this plasmid. Notable, all TEM-135 isolates, except Thai_026 (MLST ST7822) which had an Asia-type plasmid, carried the Toronto/Rio-type plasmid. As described above, Thai_026 (MLST ST7822) was the only isolate that belonged to cluster A formed by the TEM-1 isolates. Thus, the plasmid typing supported separation of this isolate from the other TEM-135 isolates, which further supports the hypothesis that TEM-135 strains have emerged from multiple independent origins. There was no TEM-135 isolate with the Africa-type plasmid. On the other hand, although all three plasmid types were found among the TEM-1 isolates, the Africa-type plasmid was the most abundant among the TEM-1 isolates (79 out of 87 isolates, 90.8%). Thus, this plasmid typing, again, implied a genetic difference of TEM-135 and TEM-1 strains. The most abundant MLST ST in TEM-1 isolates, ST1588, was strongly related to the Africa-type plasmid (53 out of 55 isolates, 96.4%). The remaining two MLST ST1588 isolates carried Toronto/Rio- and Asia-type plasmids, respectively. In total, the Africa-type plasmid was also abundant in other MLSTs, although both of the MLST ST8775 isolates had the Asia-type plasmid. Other isolates with the Asia-type plasmid were limited to MLST ST8781, MLST ST7822 (single TEM-135 isolate), MLST ST7823, and MLST ST1600 (Table 2). Interestingly, three of these MLST ST (except MLST ST1600) were linked and formed a stem in the left part of the minimum spanning tree (Fig. 2).

NG-MAST analysis. To thoroughly evaluate the genetic diversity and relatedness of the TEM-135 isolates, all PPNG isolates were also analyzed using a substantially more discriminative typing method, NG-MAST (7). The 96 PPNG isolates were divided into 58 NG-MAST STs. Each NG-MAST ST is shown in the supplemental material.

Among the four TEM-135 isolates assigned to MLST ST8788, three belonged to NG-MAST ST5134 (*porB3109* and *tbpB98*; Fig. 3A), indicating clonal dissemination. Also, four of the additional TEM-135 isolates contained highly similar *porB* alleles (Thai_098, Thai_045, Thai_003, and Thai_032) (Fig. 3B). This similarity and, accordingly, the clustering were further supported by analyzing the *tbpB* alleles of all the TEM-135 isolates (Fig. 3C). Accordingly, the NG-MAST supported the conclusion that seven of the nine TEM-135 isolates had originated from a common ancestor. Both the remaining TEM-135 isolates (Thai_026 and Thai_073) were genetically separated from this cluster by the NG-MAST (Fig. 3). This was also in full concordance with the results of the MLST and plasmid typing (Table 2 and Fig. 2).

Comparison of the Thai isolates with previously characterized Japanese PPNG isolates. Using several molecular typing methods, we tried to identify any spread of PPNG between Thailand and Japan and found the possible spread of only one TEM-1

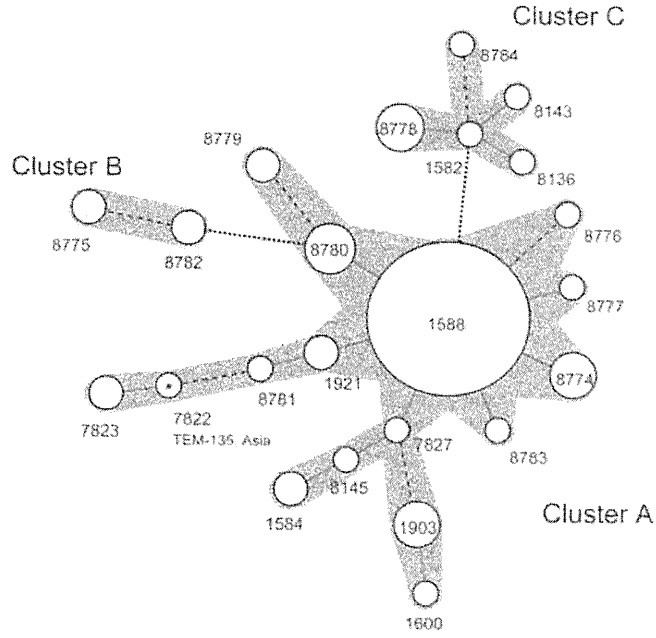

FIG 2 Minimum spanning tree analysis of multilocus sequence typing (MLST) STs observed in penicillinase-producing *N. gonorrhoeae* (PPNG) isolates cultured in Thailand in 2005 to 2007. Numbers beside the circles denote ST. Solid lines, dashed lines, and dotted lines show the interrelationship of "single-locus variant," "double-locus variant," and "triple-locus variant," respectively. The types directly or indirectly connected through single- or double-locus-variant relationships were judged to form one cluster. Each cluster is shaded gray. Sizes of circles reflect the numbers of isolates belonging to each type (for details, see text and tables). The only PPNG TEM-135 isolate belonging to cluster A (ST7822) is marked with an asterisk, and its plasmid type is given.

A

Strain	MLST	*por*	*tbpB*	NG-MAST
Thai_022, _078, _127	8778	3109	98	5134
Thai_003	8778	2809	1084	5138
Thai_045	8143	1830	1088	5132
Thai_098	8784	832	98	1292
Thai_032	1582	53	21	147
Thai_026	7822	3110	186	5135
Thai_073	8136	90	58	211

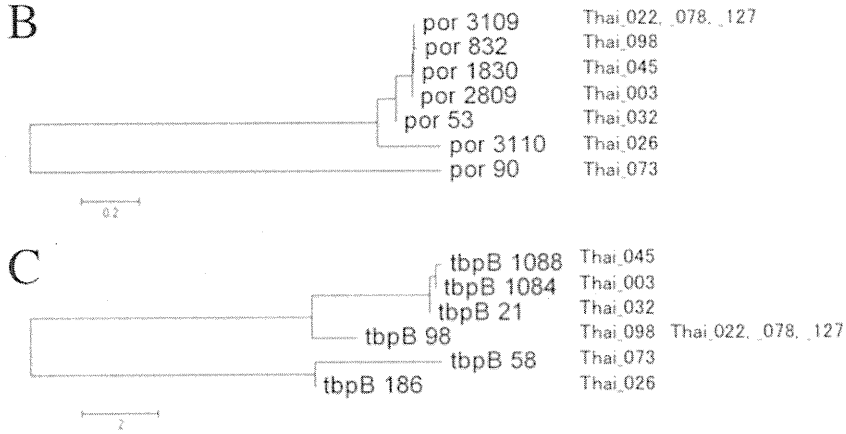

FIG 3 Molecular characterization of penicillinase-producing *N. gonorrhoeae* PPNG TEM-135 isolates. (A) Sequence types revealed by multilocus sequence typing (MLST) and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) are shown, along with *por* and *tbpB* alleles. (B and C) Neighbor-joining clustering showing similarity of *por* alleles (B) and *tbpB* alleles (C) from PPNG TEM-135 isolates.

clone. Accordingly, the previously characterized Japanese isolates NGON 08-041 and NGON 08-046 (10) and Thai_036 and Thai_093 were all, in the present study, assigned to MLST ST1584 and NG-MAST ST1478 and carried the Africa-type plasmid with bla_{TEM-1}. Despite some similarities in the MLST STs supporting, e.g., a cluster of isolates with the TEM-135 Toronto/Rio-type plasmid, no clear evidence to support international spread of any TEM-135 strains was found.

It is well-known that the β-lactamase plasmid can also easily be transferred between different *N. gonorrhoeae* strains. As the number of analyzed isolates in the present study was relatively low and they were cultured in restricted regions, Thailand (Bangkok) and Japan (Tokyo), more extensive international studies are crucial to reveal the origin and the evolutionary pathway of the TEM-135 strains, as well as the possible existence of PPNG with other TEM alleles.

Possible motive force of emergence of TEM-135. Still, the reasons and mechanisms for the emergence and dissemination of PPNG TEM-135 strains are unknown. The $bla_{TEM-135}$ allele was first found in *Salmonella enterica* serovar Typhimurium (13), and there are no major differences in the MICs of any β-lactam antimicrobials between $bla_{TEM-135}$ and bla_{TEM-1} allele-possessing isolates. The $bla_{TEM-135}$ allele has now been found in two different types of β-lactamase plasmid in PPNG, which are known to originally carry the bla_{TEM-1} allele. This fact indicates that $bla_{TEM-135}$ emerged independently in *N. gonorrhoeae* and was not acquired due to, for example, a transformational event. However, due to the similar MICs of β-lactam antimicrobials in PPNG TEM-1 and TEM-135 isolates, other factor(s) than β-lactam antimicrobial selective pressure must be the selective force in the emergence of $bla_{TEM-135}$. One possibility might be a pressure by other antibiotic(s) than penicillins. If so, we could expect some different patterns of resistance or rate of resistance to nonpenicillin antibiotics between TEM-1 and TEM-135 isolates. However, we did not observe any significant difference in those, at least when comparing susceptibility and resistance to ceftriaxone, ciprofloxacin, and tetracycline (data not shown). Another possibility is that this selective force may be an enhanced stability of the β-lactamase enzyme, which the TEM-135-specific amino acid substitution (M182T) is considered to establish (15, 21). Usually, this amino acid substitution is found in extended-spectrum TEM-type β-lactamase, as the second substitution. Since an amino acid substitution close to the active site of β-lactamase, which results in an increased MIC of cephalosporins, tends to decrease the stability of the enzyme, the M182T substitution may play a role as a stabilizer. In this context, the M182T in $bla_{TEM-135}$ in PPNG might be a prerequisite to allow the subsequent substitutions, which could extend the antimicrobial resistance spectrum of the enzyme, like several TEM-type β-lactamases found in other bacteria, e.g., TEM-20 carriers.

Necessity of monitoring TEM-135 PPNG. In conclusion, an emergence of ESBL-producing *N. gonorrhoeae* would be highly threatening to public health, because this would also be resistant to ceftriaxone, which is the first-line and last remaining option for treatment of *N. gonorrhoeae* infection in many countries world-

wide. Recently, the first *N. gonorrhoeae* strain with chromosomally mediated high-level resistance to ceftriaxone was isolated in Japan (9, 11). Although this strain was not PPNG, i.e., it had a *penA*-dependent resistance mechanism, this calls for a substantially strengthened monitoring of ceftriaxone-resistant *N. gonorrhoeae* infection and gonorrhea treatment failures, including consideration of possible emergence of ESBL-producing *N. gonorrhoeae* isolates.

In Thailand, about 10% of PPNG had TEM-135, a possible direct precursor of an ESBL. However, the prevalence and characteristics of TEM-135 strains and possible strains containing other TEM variants worldwide is unknown. This seems crucial to investigate in larger, international studies, including studies of recent geographically, phenotypically, and genetically diverse PPNG.

ACKNOWLEDGMENTS

We thank Makiko Itoh and Haruka Tsuruzono for technical assistance.

This work was partly supported by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan (grants H21-Shinkou-Ippan-001 and H23-Snikou-Shitei-020).

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2011, p. 3538–3545 Vol. 55, No. 7
0066-4804/11/$12.00 doi:10.1128/AAC.00325-11

Is *Neisseria gonorrhoeae* Initiating a Future Era of Untreatable Gonorrhea?: Detailed Characterization of the First Strain with High-Level Resistance to Ceftriaxone▽†

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Received 10 March 2011/Returned for modification 19 April 2011/Accepted 2 May 2011

Recently, the first *Neisseria gonorrhoeae* strain (H041) that is highly resistant to the extended-spectrum cephalosporin (ESC) ceftriaxone, the last remaining option for empirical first-line treatment, was isolated. We performed a detailed characterization of H041, phenotypically and genetically, to confirm the finding, examine its antimicrobial resistance (AMR), and elucidate the resistance mechanisms. H041 was examined using seven species-confirmatory tests, antibiograms (30 antimicrobials), *porB* sequencing, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), multilocus sequence typing (MLST), and sequencing of ESC resistance determinants (*penA*, *mtrR*, *penB*, *ponA*, and *pilQ*). Transformation, using appropriate recipient strains, was performed to confirm the ESC resistance determinants. H041 was assigned to serovar Bpyust, MLST sequence type (ST) ST7363, and the new NG-MAST ST4220. H041 proved highly resistant to ceftriaxone (2 to 4 μg/ml, which is 4- to 8-fold higher than any previously described isolate) and all other cephalosporins, as well as most other antimicrobials tested. A new *penA* mosaic allele caused the ceftriaxone resistance. In conclusion, *N. gonorrhoeae* has now shown its ability to also develop ceftriaxone resistance. Although the biological fitness of ceftriaxone resistance in *N. gonorrhoeae* remains unknown, *N. gonorrhoeae* may soon become a true superbug, causing untreatable gonorrhea. A reduction in the global gonorrhea burden by enhanced disease control activities, combined with wider strategies for general AMR control and enhanced understanding of the mechanisms of emergence and spread of AMR, which need to be monitored globally, and public health response plans for global (and national) perspectives are important. Ultimately, the development of new drugs for efficacious gonorrhea treatment is necessary.

Gonorrhea, caused by *Neisseria gonorrhoeae* (gonococcus), is the second-most-prevalent bacterial sexually transmitted infection globally. The disease is associated with high morbidity and socioeconomic consequences and remains a public health problem worldwide (36, 46; G. Schmid, presented at WHO/CDC symposium: Congenital syphilis and the 2005 WHO estimates of STI incidence and prevalence: using the second to help eliminate the first, 18th International Society for Sexually Transmitted Disease Research conference [ISSTDR], 28 June to 1 July 2009, London, United Kingdom). In the absence of a vaccine, appropriate diagnostics and antimicrobial therapy are the key elements for reduction and control of gonorrhea and the development of associated severe complications and sequelae, as well as further transmission of the infection (34, 36).

The treatment options, however, have diminished rapidly because of the emergence and worldwide spread of antimicrobial resistance (AMR) to all drugs previously used or considered first line, i.e., penicillins, narrow-spectrum cephalosporins, tetracyclines, macrolides, and fluoroquinolones. Furthermore, rapid emergence of resistance to spectinomycin was observed when it was widely used for treatment in the past (4), and this antimicrobial is not suitable for treatment of pharyngeal gonorrhea, nor is it currently available in many countries (3, 15, 36). Accordingly, spectinomycin is not a promising candidate for first-line empirical treatment of gonorrhea. Worryingly, in recent years, susceptibility to the currently recommended first-line antimicrobials, the extended-spectrum cephalosporins (ESCs), i.e., ceftriaxone (injectable) and cefixime (oral), has also decreased globally (3, 15, 17, 36). Furthermore, for several years, cefixime treatment failures have been recognized in Japan (9, 36, 47), where cefixime was already excluded from treatment guidelines in 2006 (36). More recently, failures have also been verified in Europe (40). However, despite the fact that susceptibility to ceftriaxone (the last remaining option for empirical first-line treatment) is decreasing globally, *in vitro* and clinical (resulting in treatment failure of urogenital gonorrhea) resistance has been lacking (3, 15, 17, 36).

Recently, the first high-level ceftriaxone-resistant gonococcal strain (H041) was isolated from the pharynx of a female commercial sex worker in Kyoto, Japan (23). H041 displayed a MIC of ceftriaxone of 2 μg/ml. This is a very high level of resistance and, previously, only one isolate having an MIC of >0.25 μg/ml (MIC = 0.5 μg/ml) (33) has been reported world-

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▽ Published ahead of print on 16 May 2011.

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wide. Unfortunately, it was not possible to definitively verify that H041 caused a treatment failure because a posttreatment isolate was not available; however, treatment failure seems likely (see Discussion). Furthermore, H041 belongs to multilocus sequence typing (MLST) sequence type (ST) ST7363 and is closely related to the successful gonococcal cefixime-resistant subclones of ST7363 (23), which are prevalent in Japan (24) and now are also being transmitted in Europe. Accordingly, H041 may be a subclone of the MLST ST7363 cefixime-resistant strains that has acquired additional resistance determinant(s), resulting in high-level ceftriaxone resistance. Historically, gonococcal AMR has mostly emerged in the World Health Organization (WHO) Western Pacific Region (WPR) and, in particular, in Japan. This resistance has spread rapidly, via sex tourists, long-distance truck drivers, and forced migration in the WHO WPR, to the Pacific Rim countries, including the United States, Southeast and Central Asia, Europe, and globally (36). The spread of ceftriaxone-resistant gonococcal strains worldwide will probably follow the same pattern. Consequently, it is crucial to examine in detail, including elucidation of the mechanisms causing the ceftriaxone resistance, the first high-level ceftriaxone-resistant gonococcal strain worldwide, recently isolated in Japan (H041) (23).

The most common mechanism in gonococci for decreased ESC susceptibility is alteration of the *penA* gene, i.e., the acquisition of a *penA* mosaic allele or alterations of amino acid A501 in the encoded penicillin-binding protein 2 (PBP2) (1, 3, 11, 14–18, 21, 23, 24, 27, 28, 32, 33, 36, 38, 40, 41, 44, 50). Mutations in the promoter and/or coding sequence of the repressor gene *mtrR* cause an overexpression of the MtrCDE efflux pump system, which further decreases ESC susceptibility (3, 11, 12, 16–18, 30, 36, 38, 43, 48, 50). Furthermore, specific *porB1b* mutations that alter amino acid G101 and A102 in the PorB1b porin (the *penB* resistance determinant) result in additional decreases in ESC susceptibility (3, 11, 16–18, 25, 26, 30, 36, 38, 50). Nevertheless, based on the relatively few studies of gonococcal isolates with decreased ESC susceptibility, polymorphisms in *ponA* (encoding PBP1) and *pilQ* (encoding the pore-forming secretin PilQ protein in the type IV pili), which both can be involved in high-level penicillin resistance, do not seem to substantially enhance the MICs of ESCs (11, 29, 45, 49, 50). At least one unknown resistance determinant exists (11, 18, 38, 50).

The aims of this study were to perform a detailed characterization, phenotypic and genetic, of the first identified high-level ceftriaxone-resistant *N. gonorrhoeae* strain (H041) worldwide in order to confirm this finding, to thoroughly examine its antimicrobial resistance, and to elucidate the ESC resistance mechanisms.

MATERIALS AND METHODS

***Neisseria gonorrhoeae* strains.** The high-level ceftriaxone-resistant strain H041 (23) and gonococcal strains ($n = 9$) selected for transformation assays to verify the resistance mechanisms, i.e., five clinical strains and four of the eight 2008 WHO *N. gonorrhoeae* reference strains (41), were included in this study. For gonococcal species verification of H041, morphology on selective culture medium, catalase and oxidase tests, microscopy after Gram staining, and seven species confirmatory tests (sugar utilization, HN-20 Rapid system identification [ID] test [Nissui, Tokyo, Japan], PhadeBact GC monoclonal test [Bactus AB, Solna, Sweden], PhadeBact GC monoclonal serovar test [Bactus AB, Solna, Sweden], MicroTrak *N. gonorrhoeae* culture confirmation test [Trinity Biotech, Wicklow, Ireland], *porA* pseudogene PCR [13], and dual-target PCR [*porA* and *opa*] [10]) were used. All strains were grown on GC culture medium as previously described (42).

Antimicrobial susceptibility testing. Ceftriaxone MIC determination was performed using the Etest method (AB bioMérieux, Solna, Sweden) according to the manufacturer's instructions. The ceftriaxone MIC of H041 was also confirmed using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) standards (8). Finally, H041 was examined for its MICs of 29 additional antimicrobials (using the Etest method) and tested with the calibrated dichotomous sensitivity (CDS) disc diffusion method (5, 35), which is used in resistance surveillance of *N. gonorrhoeae* in many countries in the WHO WPR (six antimicrobials) (Table 1). β-Lactamase production was tested using nitrocefin discs. The 2008 WHO *N. gonorrhoeae* reference strains (41) were used for quality controls in all antimicrobial susceptibility testing.

Genetic characterization. DNA was isolated in a NorDiag Bullet instrument (NorDiag ASA Company, Oslo, Norway) using a Bugs'n Beads STI-*fast* kit (NorDiag ASA Company, Oslo, Norway) according to the manufacturer's instructions. For molecular epidemiological examination, strains were genotyped by means of MLST (24), *porB* gene sequencing, and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) as described previously (39). PCR and sequencing of resistance determinants, i.e., the *penA*, *mtrR*, *porB1b*, *ponA*, and *pilQ* genes, were performed as described elsewhere (18, 41, 45).

Sequence alignments and phylogenetic analysis. Multiple-sequence alignments (nucleotide and amino acid sequences) were performed using BioEdit Sequence Alignment Editor software (version 7.0.9.0). For examination of the evolutionary relationships of H041 with other *penA* mosaic strains displaying decreased ESC susceptibility and circulating worldwide, a phylogenetic analysis using the full-length *porB* sequences in H041 and previously reported *penA* mosaic strains (11) was performed with TREECON (version 1.3b) as previously described (39).

Transformation assays. To confirm that the unique *penA* allele in H041 ($penA_{H041}$) caused the high-level resistance to ceftriaxone, the full-length $penA_{H041}$ was PCR amplified and transformed into nine recipient strains as previously described (24). These nine recipient strains displayed different molecular epidemiological sequence types, ceftriaxone MICs, and composition of ESC resistance mechanisms, such as *penA* alleles, the *mtrR* promoter, and *penB* sequences (Table 2). Briefly, the recipients were suspended in GC broth (1×10^8 cells/100 μl) and incubated with 0.2 μg of the $penA_{H041}$ PCR product (after purification using a High Pure PCR product purification kit [Roche Diagnostics GmbH, Mannheim, Germany]) for 4 h. Aliquots of 10 μl and 100 μl were inoculated on GC agar with a 4-fold higher ceftriaxone concentration than the MIC of the respective recipient. After incubation, the colonies obtained were subcultured on an antimicrobial-free GC agar plate for single-clone isolation. For confirmation, the transformation assay was performed three times for each recipient.

Nucleotide sequence accession numbers. The GenBank/EMBL/DDBJ accession numbers for the two new *penA* alleles reported in this paper are AB546858 and AB608050.

RESULTS

Phenotypic characterization of the high-level ceftriaxone-resistant *N. gonorrhoeae* strain H041. All conventional bacteriological diagnostic tests and the seven species confirmatory assays verified that H041 was a gonococcal strain, which was assigned to serovar Bpyust.

The results of the antimicrobial susceptibility testing using the Etest method (30 antimicrobials) and CDS disc diffusion method (six antimicrobials) are summarized in Table 1. Briefly, H041 was resistant to various antimicrobials, including all β-lactam antimicrobials (with possible exceptions of carbapenems, at least ertapenem and meropenem, and piperacillin-tazobactam, for which no breakpoints are available), fluoroquinolones, macrolides, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, and nitrofurantoin. The MICs of all the cephalosporins, including the recommended first-line ESCs, were very high (e.g., 2 to 4 μg/ml of ceftriaxone and 8 μg/ml of cefixime). H041 did not produce any β-lacta-

TABLE 1. MIC using the Etest method and zone sizes with the calibrated dichotomous sensitivity disc diffusion method of *Neisseria gonorrhoeae* H041 to various antimicrobials

Antimicrobial	Class	MIC Etest result in μg/ml (agar dilution result), interpretation[a,b]	CDS (mm)[c]
Penicillin G	β-Lactams, penicillins	4, R[a,b]	0
Ampicillin		2	ND
Amdinocillin		>256	ND
Piperacillin-tazobactam		0.25	ND
Aztreonam	β-Lactam, monobactam	128	ND
Cefuroxime	β-Lactams, cephalosporins	16, R[a]	ND
Cefpodoxime		16, R[a]	3
Ceftazidime		16, R[a]	ND
Cefotaxime		8, R[a,b]	ND
Cefixime		8 (8), R[a,b]	ND
Ceftriaxone		4 (2), R[a,b]	1
Cefepime		16, R[a]	ND
Ertapenem	β-Lactams, carbapenems	0.064	ND
Meropenem		0.125	ND
Imipenem		2	ND
Ciprofloxacin	Fluoroquinolones	>32, R[a,b]	1
Levofloxacin		>32	ND
Moxifloxacin		6	ND
Azithromycin	Macrolides	1, R[b]	ND
Erythromycin		2	ND
Gentamicin	Aminoglycosides	4	ND
Kanamycin		16	ND
Tobramycin		6	ND
Spectinomycin	Aminocyclitol	16, S[a,b]	9
Tetracycline	Tetracycline	4, R[b]	5
Tigecycline	Glycylcycline	0.5	ND
Trimetoprim-sulfamethoxazole	Folic acid antagonists	1	ND
Chloramphenicol	—	4	ND
Nitrofurantoin	—	4	ND
Rifampin	—	0.25	ND

[a] Antimicrobial susceptibility testing was performed using the Etest method (AB bioMérieux, Solna, Sweden) on all antimicrobials according to the instructions from the manufacturer (results were rounded up to whole MIC dilutions). Furthermore, agar dilution was additionally performed for ceftriaxone and cefixime (in parentheses) according to the method described by the Clinical Laboratory and Standards Institute (CLSI) (8). Where available, interpretative criteria (S, susceptible; I, intermediate; R, resistant) from the Clinical and Laboratory Standards Institute (CLSI) (8) were used.

[b] Interpretative criteria (S, I, R) from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Available online at: http://eucast.org/ (Accessed 30 April, 2011).

[c] The calibrated dichotomous sensitivity (CDS) disc diffusion method (5, 35) is used for antimicrobial resistance testing in many countries in the World Health Organization (WHO) Western Pacific Region. ND, not determined.

mase. The strain was, however, susceptible to spectinomycin and rifampin. Furthermore, the MICs of aminoglycosides and tigecycline were also relatively low (no breakpoints are available for these antimicrobials). The CDS disc diffusion method also identified H041 as resistant to ceftriaxone, cefpodoxime, penicillin G, tetracycline, and ciprofloxacin but susceptible to spectinomycin (Table 1).

Genetic characterization and elucidation of the mechanisms causing the high-level ceftriaxone resistance in *N. gonorrhoeae* strain H041. The molecular epidemiological characterization assigned H041 as MLST ST7363 and as the not-previously described NG-MAST sequence type ST4220 (www.ng-mast.net). A phylogenetic analysis using *porB* sequences showed that H041 is closely related to other *penA* mosaic strains with decreased ESC susceptibility that have been shown to circulate worldwide (Fig. 1).

The sequencing of ESC resistance determinants showed that H041 possessed a unique *penA* mosaic allele ($penA_{H041}$) and the previously described *mtrR*, *penB*, and *ponA1* (L421P) resistance determinants. No new *pilQ* mutations were found. Thus, the only new resistance determinant, which consequently was suspected to cause the high ESC MICs, was $penA_{H041}$ (GenBank accession number AB546858). $penA_{H041}$ was highly similar (i.e., 97.6% nucleotide identity and only 12 PBP2 amino acid differences that clustered in two regions) to the previously described *penA* mosaic allele X that has been correlated with cefixime treatment failures in Japan. Of these 12 amino acids, five were unique compared with any gonococcal PBP2 sequence previously described, but one of these (I486) has been found in *Neisseria meningitidis* and *Neisseria flavescens* (Fig. 2). Accordingly, $penA_{H041}$ contained only four PBP2 amino acid residues that have not been previously reported in any *Neisseria* species; compared to *penA* mosaic X, these consisted of A311V, T316P, A328T, and T484S (Fig. 2).

Transformation assays confirm that the unique $penA_{H041}$ caused the high-level resistance to ceftriaxone and other extended-spectrum cephalosporins. Based on their different genotypes, ceftriaxone MICs, and composition of ESC resistance determinants, nine strains were selected as recipients of $penA_{H041}$ in transformation assays (Table 2).

Upon transformation with $penA_{H041}$, the ceftriaxone MICs of the recipients increased to 0.125 to 8 μg/ml, i.e., by 16- to 500-fold. Accordingly, the ceftriaxone MICs of all recipients, with the exception of NG9901 (0.125 μg/ml), increased above

TABLE 2. *Neisseria gonorrhoeae* strains with different ceftriaxone MICs and containing divergent genetic ceftriaxone resistance mechanisms that were used as recipients in transformation of the full-length *penA* gene from H041

Strain	MLST[a]	NG-MAST[b]	Ceftriaxone MIC (μg/ml)[c]	*penA*[d] (allele)	*mtrR*[e]	*penB*[f]	*ponA*[g]
NG9901	ST7363	ST240	<0.002	*penA* XXXVI (mosaic[h])	WT	WT	WT
WHO F	NEW	ST3303	<0.002	*penA* XV (WT)	WT	WT	WT
NG9903	ST7359	ST4058	0.004	*penA* II (A345a)	WT	WT	WT
NG9807	ST7363	ST4093	0.016	*penA* II (A345a)	A-del	Yes	L421P
WHO M	ST7367	ST3304	0.016	*penA* II (A345a)	A-del	Yes	L421P
WHO K	ST7363	ST1424	0.064	*penA* X (mosaic)	A-del	Yes	L421P
NG0003	ST7363	ST4068	0.125	*penA* X (mosaic)	A-del	Yes	L421P
35/02	ST7363	ST326	0.125	*penA* XXVIII (mosaic)	A-del	Yes	L421P
WHO L	ST1590	ST1422	0.125	*penA* VII (A501V)	A-del	Yes	L421P

[a] MLST, multilocus sequencing typing (24).
[b] NG-MAST, *Neisseria gonorrhoeae* multiantigen sequence typing (39).
[c] Etest results were rounded up to whole MIC steps. MICs of <0.002 μg/ml were calculated as 0.001 μg/ml in the MIC ratios in Fig. 3.
[d] The *penA* mosaic allele encodes a mosaic penicillin binding protein 2 (PBP2) that causes a decreased susceptibility to extended-spectrum cephalosporins. Mosaic X has been found in cefixime-resistant *N. gonorrhoeae* isolates in Japan (18, 36, 50).
[e] A-del indicates a characteristic single nucleotide (A) deletion in the inverted repeat of the promoter region of *mtrR* that causes an overexpression of the MtrCDE efflux pump that results in a further decrease in susceptibility to ESCs (18, 36, 50).
[f] "Yes" indicates the presence of the alterations of amino acids 120 and 121 in porin PorB (*penB* alteration) that cause a decreased intake of ESCs and, accordingly, a further decrease in susceptibility to ESCs (18, 36, 50). WT, wild type.
[g] The alteration of amino acid 421 in PBP1 (encoded by *penA*) causes a decreased susceptibility to penicillins (18, 29, 50).
[h] *penA* mosaic allele that has not been previously described and whose sequence has been deposited in GenBank with accession number AB608050.

the resistance breakpoint (>0.25 μg/ml) (8) independent of other resistance determinants. Remarkably, WHO F, which has wild-type alleles of all ESC and penicillin resistance determinants, displayed a ceftriaxone MIC of 0.5 μg/ml after transformation (500-fold MIC increase) (Fig. 3).

All single-clone transformants (derived from all recipient strains) showed *mtrR*, *penB*, and *ponA* sequences identical to those in the recipients. All the single-clone transformants also contained the $penA_{H041}$ allele. In most transformants, the transformed $penA_{H041}$ sequence was identical to the sequence in H041. However, in a few transformants, such as those derived from the WHO F and WHO M strains, some point mutations differed from the $penA_{H041}$ sequence. These were considered to represent spontaneous mutations, mutations in junctions for recombination and/or belonging to the *penA* allele of the recipient. The majority of these mutations were nonsynonymous, and none was located in any segment of the mosaic *penA* allele affecting the ceftriaxone MICs. Consequently, the transformation experiments confirmed that $penA_{H041}$ was the cause of the high-level ceftriaxone resistance.

DISCUSSION

The present study describes the detailed phenotypic and genetic confirmation and characterization, including elucidation of the resistance mechanisms, of the first identified *N. gonorrhoeae* strain (H041) displaying high-level resistance to ceftriaxone worldwide. H041 was isolated from a female commercial sex worker in Japan (23), and the ceftriaxone MIC of H041 was 4- to 8-fold higher than any previously observed. Ceftriaxone is also the last remaining option for empirical first-line treatment of gonorrhea. Accordingly, *N. gonorrhoeae* has now shown its ability to develop resistance to ceftriaxone also and, although the biological fitness of ceftriaxone resistance in *N. gonorrhoeae* remains unknown, the gonococcus may become a true superbug that initiates a future era of untreatable gonorrhea.

Although a posttreatment isolate was unavailable (only one specimen positive with SDA [ProbeTec ET; Becton-Dickinson], sampled 2 weeks after treatment) to definitively verify treatment failure using 1 g ceftriaxone intravenously (23), it seems likely that this was the first gonorrhea clinical failure caused solely by high resistance of the bacteria to ceftriaxone, based on the posttreatment positive-SDA sample (all residual gonococcal DNA is expected to be eliminated before 2 weeks posttreatment) (2), the very high ceftriaxone MIC, and all available data regarding pharmacodynamic parameters for ESCs. Thus, according to Monte Carlo simulations, the 1 g ceftriaxone intravenously that was used for treatment (in full concordance with treatment recommendations for urogenital and pharyngeal gonorrhea in the Japanese treatment guidelines) results in median times of free ceftriaxone above the MIC ($fT_{>MIC}$) of only 6.0 h (0 to 20.3 h) and 0 h (0 to 5.6 h) for the detected MICs of 2 μg/ml (agar dilution method) and 4 μg/ml (Etest method), respectively (6). Accordingly, using 1 g ceftriaxone for treatment, the ceftriaxone MIC of H041 will make the strain escape eradication in most (if not all) patients. Furthermore, this was a case of pharyngeal gonorrhea, which is substantially harder to treat than urogenital gonorrhea (3, 36), and the infection probably resolved spontaneously within 3 months. Nevertheless, despite the fact that a clinical history was recorded, re-reinfection cannot be completely excluded, especially as the patient was a commercial sex worker.

The resistance determinants causing the high ESC MICs in H041 were also elucidated. The unique $penA_{H041}$ mosaic allele was found to be responsible; upon transformation of $penA_{H041}$ into recipients with different ESC MICs and resistance mechanisms, their ceftriaxone MICs increased to 0.125 to 8 μg/ml, i.e., by 16-fold to 500-fold. Nevertheless, additional resistance determinants, especially *mtrR* and *penB* (and "factor X," i.e., the still unidentified determinant), were needed to reach the same level of ceftriaxone MIC as H041, a synergy that was previously reported (18, 36, 50). Factor X was not transformable using the H041 genome (data not shown), which has also

FIG. 1. Phylogenetic tree describing the evolutionary relationships of full-length *porB* gene sequences of the high-level ceftriaxone-resistant *Neisseria gonorrhoeae* strain H041 compared with those of previously published *N. gonorrhoeae penA* mosaic isolates (11). The 2008 WHO K reference strain (41), containing a *penA* mosaic allele X and cultured in Japan in 2001, was used to root the tree. The *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) sequence type (ST) and number of isolates are indicated.

previously been described using other gonococcal genomes (18, 50). $penA_{H041}$ is highly similar to the previously described *penA* mosaic allele X (causing ceftriaxone MICs of only 0.064 to 0.125) (Table 2), which has been correlated with cefixime treatment failures in Japan, having only 12 PBP2 amino acid differences clustering in two regions. Of these 12 amino acids, only four have not been previously reported in any *Neisseria* species; compared with *penA* mosaic X, these consist of A311V, T316P, A328T (in region A), and T484S (in region B) (Fig. 2). It was also confirmed that transformation of only the $penA_{H041}$ region A into the recipients caused, for most, as high a ceftriaxone MIC as transforming the full-length $penA_{H041}$ (data not shown). Although further confirmatory studies are needed, it is highly probable that A311V and T316S are the alterations causing the high ceftriaxone resistance, i.e., due to the proximity to the β-lactam active site in PBP2. Despite this fact, the MICs of some β-lactam antimicrobials, such as penicillins (especially piperacillin-tazobactam) and carbapenems (particularly ertapenem and meropenem), were surprisingly low. $penA_{H041}$ could also easily be transformed to other gono-