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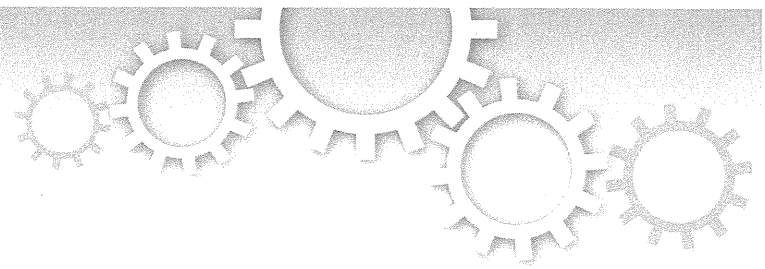

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Superiority of pulmonary administration of mepenzolate bromide over other routes as treatment for chronic obstructive pulmonary disease

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We recently proposed that mepenzolate bromide (mepenzolate) would be therapeutically effective against chronic obstructive pulmonary disease (COPD) due to its both anti-inflammatory and bronchodilatory activities. In this study, we examined the benefits and adverse effects associated with different routes of mepenzolate administration in mice. Oral administration of mepenzolate caused not only bronchodilation but also decreased the severity of elastase-induced pulmonary emphysema; however, compared with the intratracheal route of administration, about 5000 times higher dose was required to achieve this effect. Intravenously or intrarectally administered mepenzolate also showed these pharmacological effects. The intratracheal route of mepenzolate administration, but not other routes, resulted in protective effects against elastase-induced pulmonary damage and bronchodilation at a much lower dose than that which affected defecation and heart rate. These results suggest that the pulmonary route of mepenzolate administration may be superior to other routes (oral, intravenous or intrarectal) to treat COPD patients.

Chronic obstructive pulmonary disease (COPD) is a serious health problem and the most important etiologic factor of which is cigarette smoke (CS). COPD is currently the fourth leading cause of death in the world and its prevalence and mortality rates are steadily increasing[1]. This disease state is defined by a progressive and not fully reversible airflow limitation associated with an abnormal inflammatory response-mediated permanent enlargement of the pulmonary airspace[1–3]. Thus, for the clinical treatment of COPD, it is important not only to improve the airflow limitation by bronchodilation, but also to suppress disease progression by controlling inflammatory processes.

Bronchodilators (β_2-agonists and muscarinic antagonists) are currently used for the treatment of COPD owing to their ameliorating effects on airflow limitation[2,4,5]. Steroids are also used to suppress inflammatory processes in COPD patients; however steroids do not significantly modulate disease progression or mortality[5,6], because the inflammation associated with COPD tends to be resistant to steroid treatment[7]. Thus, the development of new types of anti-inflammatory drugs to treat COPD is paramount.

The number of drugs reaching the marketplace each year is decreasing, mainly due to the unexpected adverse effects of potential drugs being revealed at advanced clinical trial stages. For this reason, we proposed a new strategy for drug discovery and development (drug re-positioning)[8]. In this strategy, compounds with therapeutically beneficial activity are screened from a library of approved medicines to be developed for new indications. The advantage of this approach is that there is a decreased risk for unexpected adverse effects in humans because the safety aspects of these drugs have already been well characterized in humans[8]. From a library of approved medicines, we screened compounds that prevent elastase-induced pulmonary emphysema in mice, and selected mepenzolate bromide (mepenzolate)[9], which is an orally administered muscarinic receptor antagonist used to treat gastrointestinal disorders (such as peptic ulcers and irritable bowel syndrome)[10–12]. We showed that mepenzolate not only exerts an anti-inflammatory effect via a muscarinic receptor-independent mechanism, but also a bronchodilatory effect via a muscarinic receptor-dependent mechanism[9].

Oxidative stress, such as superoxide anion, is believed to play a major role in abnormal inflammation in COPD patients and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase plays an important role in the production of superoxide anions[13]. The body contains a number of endogenous anti-oxidant proteins such as superoxide dismutase and glutathione S-transferase, with a decrease in these proteins reported to be involved in the pathogenesis of COPD[14,15]. We reported that mepenzolate not only suppressed the elastase-induced production of superoxide anions and NADPH oxidase activation but also stimulated the expression of superoxide dismutase and glutathione S-transferase, suggesting that mepenzolate suppresses elastase-induced pulmonary emphysema via decrease of oxidative stress[9]. Based on these results, we proposed that mepenzolate could serve as a candidate drug for the treatment of COPD.

The route of administration of each particular drug is an important factor to be taken into account when considering its final clinical application. Most muscarinic receptor antagonists currently used for treating COPD patients are administered via the lung[16] because the systemic administration of this type of drug frequently results in adverse effects on cardiac and intestinal functions (such as arrhythmia, heart palpitations and constipation). In this way, we chose the pulmonary route of mepenzolate administration (intratracheal administration or inhalation) in our previous study on mice[9]. On the other hand, since mepenzolate was approved for use as an orally administered drug, the development of this drug to be taken orally for COPD would be more convenient compared to other administration routes. Thus, to determine the appropriate route of mepenzolate administration for possible use by COPD patients, we examined here the effect of different administration routes on this drug's beneficial and adverse effects in mice. When administered intratracheally, mepenzolate showed protective effects on elastase-induced pulmonary damage at a much lower dose than that which affected fecal pellet output and heart rate. With respect to the other administration routes (oral, intravenous and intrarectal), mepenzolate showed protective and adverse effects at similar doses. These results suggest that the pulmonary administration route for mepenzolate may be superior to other routes to treat COPD patients.

Results

Effect of different administration routes of mepenzolate on pulmonary damage and airway resistance. We recently reported that the intratracheal administration or inhalation of mepenzolate suppressed porcine pancreatic elastase (PPE)-induced inflammatory responses, pulmonary emphysema, alteration of lung mechanics, and respiratory dysfunction[9]. As a first step in the present study, we confirmed these effects of intratracheally administered mepenzolate.

As shown in Fig. 1a, the total number of leucocytes and the individual number of neutrophils in bronchoalveolar lavage fluid (BALF), which serve as indicators of pulmonary inflammatory responses, increased after the PPE treatment; this increase was partially suppressed by the simultaneous intratracheal administration of mepenzolate (38 or 190 μg/kg). Histopathological analysis revealed that while PPE administration damaged the alveolar walls and increased mean linear intercept (MLI), this effect could again be partly suppressed by the administration of mepenzolate (38–940 μg/kg; Fig. 1b and c). The alteration of lung mechanics associated with pulmonary emphysema is characterized by a decrease in elastance[17]. PPE treatment decreased both total respiratory system elastance (whole lung elastance, including the bronchi, bronchioles and alveoli) and tissue elastance (elastance of alveoli), both of which were partially restored by simultaneous mepenzolate administration (Fig. 1d). PPE treatment also decreased the $FEV_{0.05}/FVC$ ratio (Fig. 1d), which is homologous to the FEV_1/FVC ratio in humans[18,19]. Mepenzolate administration restored the $FEV_{0.05}/FVC$ ratio towards control values (Fig. 1d). The bronchodilation activity exerted by mepenzolate was monitored by its inhibitory effect on the increase in airway resistance induced by methacholine[9]. As shown in Fig. 1e, the methacholine-induced increase in airway resistance was completely suppressed by the intratracheal administration of mepenzolate, with the dose required to decrease the airway resistance (0.3 μg/kg) being much lower than that required to protect the pulmonary tissue against PPE-induced damage (38 μg/kg, Fig. 1c). The results in Fig. 1 are thus consistent with those reported previously[9].

We subsequently examined the effects of orally administered mepenzolate on the same parameters as those described above. As shown in Fig. 2a–c, orally administered mepenzolate protected against PPE-induced inflammatory responses and pulmonary emphysema; however, the dose required to achieve this protective effect (190 mg/kg) was much higher than that found when the drug was administered intratracheally (Fig. 1a–c). Orally administered mepenzolate also suppressed PPE-induced alterations of lung mechanics but did not significantly affect respiratory dysfunction (Fig. 2d). The bronchodilatory effect of orally administered mepenzolate was also observed only at higher doses (Fig. 2e) compared with that obtained with intratracheal mepenzolate administration (Fig. 1e). Furthermore, in contrast to the results for intratracheal administration, orally administered mepenzolate showed both bronchodilatory and protective effects against PPE-induced pulmonary disorders at roughly similar doses (Fig. 2).

We also examined the effects of intravenously administered mepenzolate. As shown in Fig. 3a–c, this route of mepenzolate administration (10 μg/kg) protected against PPE-induced inflammatory responses and pulmonary emphysema. Compared to the intratracheal administration, although the effective dose was slightly lower via the intravenous route, the extent of amelioration was not as apparent (Fig. 3a–c). Furthermore, intravenous administration of the highest dose of mepenzolate tested for this route (100 μg/kg) did not protect against PPE-induced pulmonary damage (Fig. 3a and c), nor did it significantly restore the lung mechanics and respiratory function, both of which were affected by the PPE treatment (Fig. 3d). These results demonstrate that intravenously administered mepenzolate is not as effective against PPE-induced pulmonary damage as that achieved via the intratracheally administered route. On the other hand, almost complete inhibition of the methacholine-induced increase in airway resistance was observed with the intravenous administration of mepenzolate (Fig. 3e). These results suggest that the protective effects of mepenzolate against PPE-induced pulmonary damage and its bronchodilatory effect are independent of each other.

Monitoring of the mepenzolate level in blood and tissue after administration of the drug via different routes. High performance liquid chromatography (HPLC) analysis was used to determine the level of mepenzolate in plasma and tissue. We initially examined the plasma level of mepenzolate after its intravenous administration, with the detected levels of the drug increasing in a dose-dependent manner (Fig. 4a). Examination of the time-course profile showed that mepenzolate was clearly detectable at 1 min, significantly reduced after 5 min, and undetectable 30 min following its intravenous administration (Fig. 4b), suggesting that mepenzolate is very unstable in blood. We then performed similar analyses to determine plasma mepenzolate levels after oral administration of the drug. As shown in Fig. 4c, mepenzolate could be detected in the plasma only when a very high dose (940 mg/kg) of the drug was administered via this route. Furthermore, the peak level was achieved 30 min after oral administration (Fig. 4d). In contrast, when mepenzolate was administered via the intratracheal route, it could be detected at a relatively lower dose (10 mg/kg) (Fig. 4e). Furthermore, the detection was very rapidly (at 1 min) (Fig. 4f). These results suggests that the efficiency of absorption into the circulation is higher for the intratracheal route of administration than the oral route. We also tried to detect mepenzolate in the lung tissue of treated mice, with the drug detected following

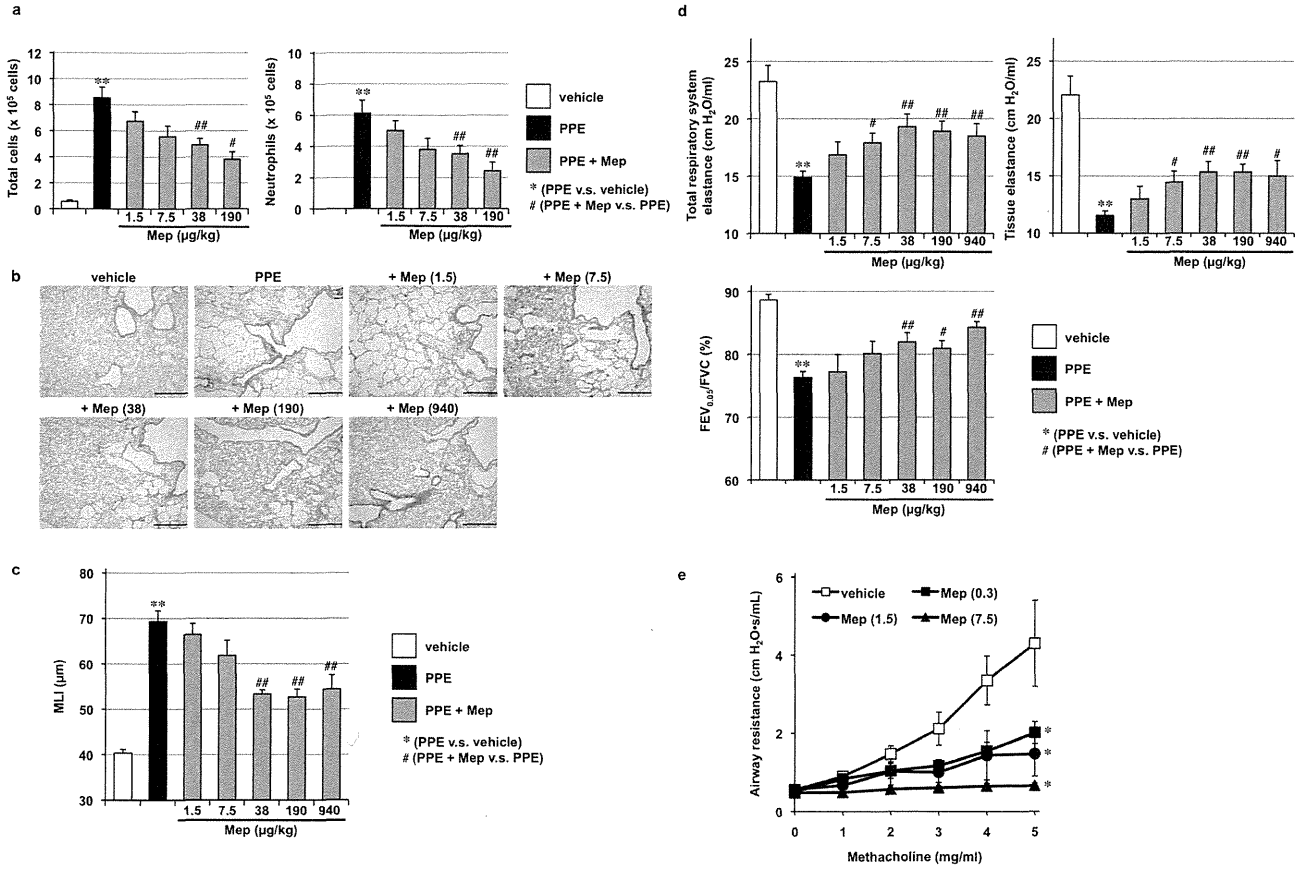

Figure 1 | Effect of intratracheal administration of mepenzolate on PPE-induced pulmonary damage and methacholine-induced airway constriction. Mice were treated with or without (vehicle) PPE (15 U/kg) once only on day 0 (a–d). The indicated doses (μg/kg) of mepenzolate (Mep) were administered intratracheally once only (a) or once daily for 12 days (from day 0 to day 11) (b–d). Twenty-four hours after the PPE administration, BALF was prepared and the total cell number and the number of neutrophils were determined as described in the Materials and Methods (a). Sections of pulmonary tissue were prepared on day 14 and subjected to histopathological examination (H & E staining) (scale bar, 500 μm) (b). Airspace size was estimated by determining the MLI as described in the Materials and Methods (c). Total respiratory system elastance, tissue elastance, and $FEV_{0.05}$/FVC were determined on day 14 as described in the Materials and Methods (d). Indicated doses (μg/kg) of mepenzolate (Mep) were administered intratracheally. After 1 h, mice were exposed to nebulized methacholine 5 times and airway resistance was determined after each methacholine challenge as described in the Materials and Methods (e). Values represent mean ± S.E.M. (n = 3–10). * or $^{\#}$ $P < 0.05$; ** or $^{\#\#}$ $P < 0.01$.

administration via the intratracheal route (Fig. 4g), but not for orally or intravenously administered drug (data not shown). The results in Fig. 4g also showed that most of intratracheally administered mepenzolate disappeared from the lung within 30 min.

Effect of intrarectally administered mepenzolate on pulmonary damage and airway resistance. It has been reported that, compared to the oral route of administration, the intrarectal route for some drugs results in a much higher uptake efficiency into the circulation due to the circumvention of drug inactivation within the gastrointestinal tract and the first-pass effect, or the higher efficiency of absorption via the rectum compared with the small intestine[20,21]. For these reasons, we examined the effect of intrarectally administered mepenzolate on PPE-induced pulmonary damage and airway resistance. As shown in Fig. 5a–c, intrarectally administered mepenzolate showed a protective effect against PPE-induced pulmonary damage at doses of 1.5 or 7.5 mg/kg, which are much lower than that required in the case of oral administration (Fig. 2a–c). Similar results were observed with respect to the PPE-induced alteration of lung mechanics and respiratory dysfunction; however, the amelioration of respiratory function by intrarectally administered mepenzolate was not statistically significant (Fig. 5d). As shown in Fig. 5e, intrarectally administered mepenzolate suppressed the methacholine-induced increase in airway resistance at lower doses to that seen in response to oral administration of the drug (Fig. 2e).

We also determined the plasma level of mepenzolate after the intrarectal administration of this drug. The dose-response and time-course profiles (Fig. 5f and g) revealed that the absorption into the circulation of intrarectally administered mepenzolate is much more efficient and rapid than that seen with orally administered drug (Fig. 4c and d). The results in Fig. 5 thus suggest that the intrarectal route of mepenzolate administration is more effective than the oral route due to the lower effective doses required.

We also examined the effect of different routes of mepenzolate administration on CS-induced lung inflammatory responses. As shown in Fig. 6a, the total number of leucocytes and the individual number of macrophages in BALF increased after the CS treatment and this increase was suppressed by the simultaneous intratracheal administration of mepenzolate (38 or 190 μg/kg). Similar suppression was observed with oral, intravenous or intrarectal administration of mepenzolate (Fig. 6b–d), however, the oral administration required much higher dose of mepenzolate than the intratracheal administration (Fig. 6a, b). Furthermore, the extent of suppression was not so apparent with the intravenous or intrarectal administration as the intratracheal administration and the suppression of

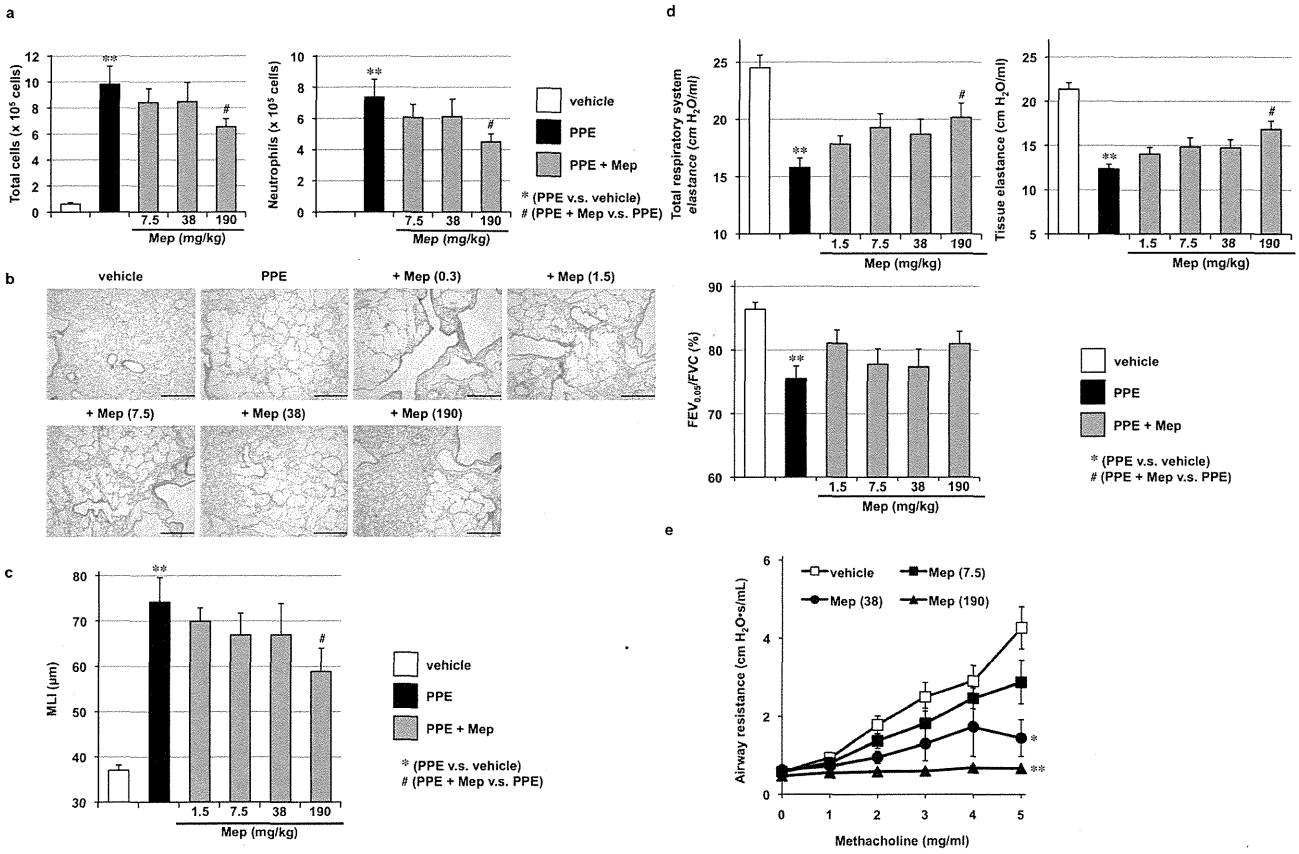

Figure 2 | Effect of oral administration of mepenzolate on PPE-induced pulmonary damage and methacholine-induced airway constriction. Administration of PPE, mepenzolate and methacholine was performed as described in the legend of Fig. 1, except that mepenzolate was administered orally (a–e). Analysis of inflammatory responses (a), histopathological examination (scale bar, 500 μm) (b), determination of the MLI (c), measurement of lung mechanics and respiratory function (d) and measurement of airway resistance (e) were carried out as described in the legend of Fig. 1. Values represent mean ± S.E.M. (n = 3–8). * or # $P < 0.05$; ** $P < 0.01$.

increase in the total number of leucocytes and the individual number of macrophages in BALF by intravenous administration of mepenzolate (10 or 100 μg/kg) was not statistically significant (Fig. 6).

Effect of different administration routes of mepenzolate on the appearance of adverse effects. To determine the appropriate administration route of any drug, it is important to consider not only its beneficial but also its adverse side-effects. For the clinical application of mepenzolate to treat COPD patients, both constipation and arrhythmia (heart palpitations) have been noted as adverse side-effects that occur due to the inhibitory effects of this drug on the muscarinic receptor and the resulting inhibition of intestinal motility and increased heart rate[22,23]. We therefore examined the effect of different routes of mepenzolate administration on defecation and heart rate in treated mice.

Mice were subjected to restraint stress as a means to increase fecal pellet output. As shown in Fig. 7, mepenzolate administration suppressed fecal pellet output with respect to control (untreated) mice for each of the routes tested. Compared to the protective effects exerted by mepenzolate against PPE-induced pulmonary damage (Fig. 1), doses administered via the intratracheal administration route that were more than 100 times higher were required to affect fecal pellet output (Fig. 7a). In contrast, less than one hundredth the dose of mepenzolate required to provide a protective effect against lung damage significantly affected fecal pellet output when the oral administration route was used (Fig. 7b). As for the intravenous or intrarectal routes of administration, roughly similar doses of mepenzolate were required for both inhibition of fecal pellet output and protection against PPE-induced pulmonary damage (Figs. 3c, 5c, 7c and 7d). These results suggest that intratracheally administered mepenzolate could protect against PPE-induced pulmonary damage without affecting gut motility. Moreover, the results also suggest that orally administered mepenzolate affects gut motility directly (but not after absorption), because the dose required to suppress fecal pellet output was much lower compared to that required for other pharmacological effects.

Lastly, we examined the effect of mepenzolate on heart rate as measured by infrared sensor. As shown in Fig. 8a, intratracheally administered mepenzolate increased heart rate only at a dose that was much higher than that required to protect against PPE-induced pulmonary damage (Fig. 1c). On the other hand, the oral, intravenous or intrarectal routes of mepenzolate administration increased the heart rate at doses roughly similar to that required for pulmonary protection (Figs. 2c, 3c, 5c, 8b–d). These results suggest that intratracheally administered mepenzolate protects against PPE-induced pulmonary damage without affecting heart rate.

Discussion

Since COPD is characterized by airflow limitation and abnormal inflammatory responses, a combination of anti-inflammatory drugs (such as steroids) and bronchodilators is the standard treatment regime[24,25]. Since mepenzolate has both anti-inflammatory and bronchodilatory activities, this drug may be beneficial for treating COPD without the concomitant use of other drugs. In particular, the anti-inflammatory effect of mepenzolate is an important property of this drug, because the inflammation associated with COPD tends to

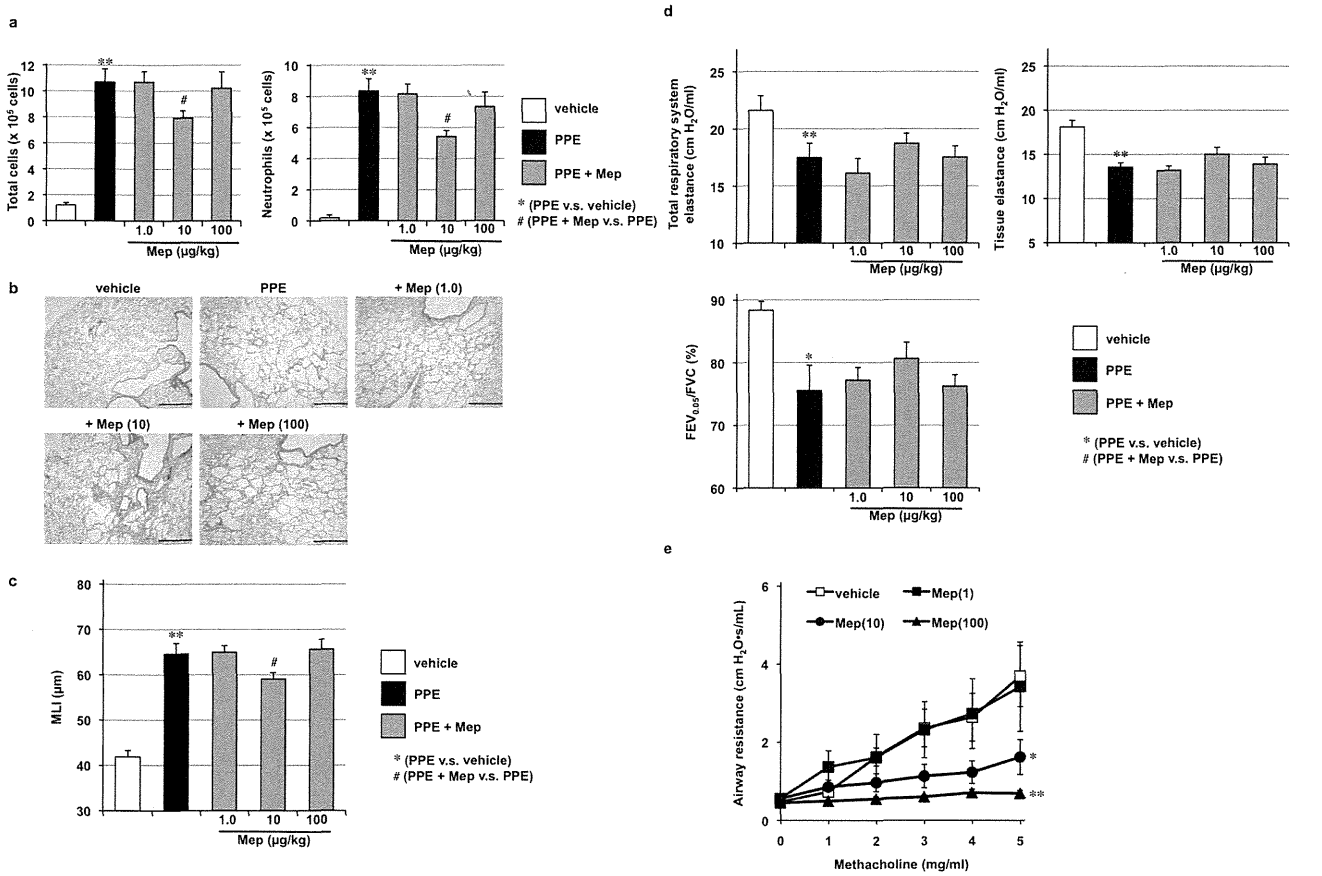

Figure 3 | Effect of intravenous administration of mepenzolate on PPE-induced pulmonary damage and methacholine-induced airway constriction. Administration of PPE, mepenzolate and methacholine was performed as described in the legend of Fig. 1, except that mepenzolate was administered intravenously (a–e). Analysis of inflammatory responses (a), histopathological examination (scale bar, 500 μm) (b), determination of the MLI (c), measurement of lung mechanics and respiratory function (d) and measurement of airway resistance (e) were carried out as described in the legend of Fig. 1. Values represent mean ± S.E.M. (n = 3–14). * or # $P < 0.05$; ** $P < 0.01$.

show resistance to steroid treatment; common steroids as such do not significantly modulate disease progression and mortality[5–7]. This insensitivity to steroids can be explained by the notion that steroids suppress the expression of pro-inflammatory genes via their action on histone deacetylase (HDAC) 2[26,27]. CS also inhibits the activity and expression of HDAC2[26]. On the other hand, mepenzolate can restore HDAC activity under inflammatory conditions[9], which may explain its superior anti-inflammatory activity to steroids under these conditions (see below). In an animal model of elastase-induced lung inflammation and emphysema, we reported that steroids do not provide protective or therapeutic benefits against PPE-induced pulmonary emphysema, alterations of lung mechanics, or respiratory dysfunction[19], whereas mepenzolate was effective against these disorders under the same experimental conditions[9]. Based on these results, we considered that mepenzolate could be therapeutically beneficial to treat COPD patients, which motivated us to examine here the effect of different routes of mepenzolate administration (intratracheal, oral, intravenous or intrarectal) on its beneficial effects (protection against PPE-induced pulmonary damage and bronchodilation) and adverse side-effects (alteration of gut motility and heart rate) in mice.

Intratracheally administered mepenzolate protected against PPE-induced pulmonary damage (inflammatory responses, pulmonary emphysema, alteration of lung mechanics and respiratory dysfunction) at a dose of 38 μg/kg and showed bronchodilation activity at a dose of 0.3 μg/kg, as reported recently[9]. We here found that this mode of administration required a much higher dose (4.7 mg/kg) to affect fecal pellet output and heart rate, thus demonstrating that intratracheally administered mepenzolate could suppress PPE-induced pulmonary damage and improve airflow limitation without affecting these other parameters, which is of particular clinical significance in terms of the use of this drug to treat COPD patients. This may be due to the fact that intratracheally administered mepenzolate is localized within the lung, in contrast to the other routes of administration studied. Furthermore, the lower dose of mepenzolate required for bronchodilation (compared to protection against PPE-induced pulmonary damage) suggests that intratracheally administered mepenzolate is localized within the bronchi rather than the alveoli, because such differences in dosage were not observed for the other forms of systemic administration.

We found here that the oral and intravenous routes of mepenzolate administration also protected against PPE-induced pulmonary damage and showed bronchodilatory activity. However, the improvement of respiratory function ($FEV_{0.05}$/FVC) by mepenzolate was not statistically significant when the drug was administered via these routes. Compared to intravenous or intratracheal administration, much higher doses of mepenzolate were required to protect against PPE-induced pulmonary damage for the oral route of administration, suggesting that the efficiency of absorption into the circulation is very poor for administration via this route. It should be noted that mepenzolate achieved beneficial and adverse effects at roughly similar doses when administered orally or intravenously (except for the effect of orally administered mepenzolate on fecal pellet output). When the route of administration was intrarectal rather than oral,

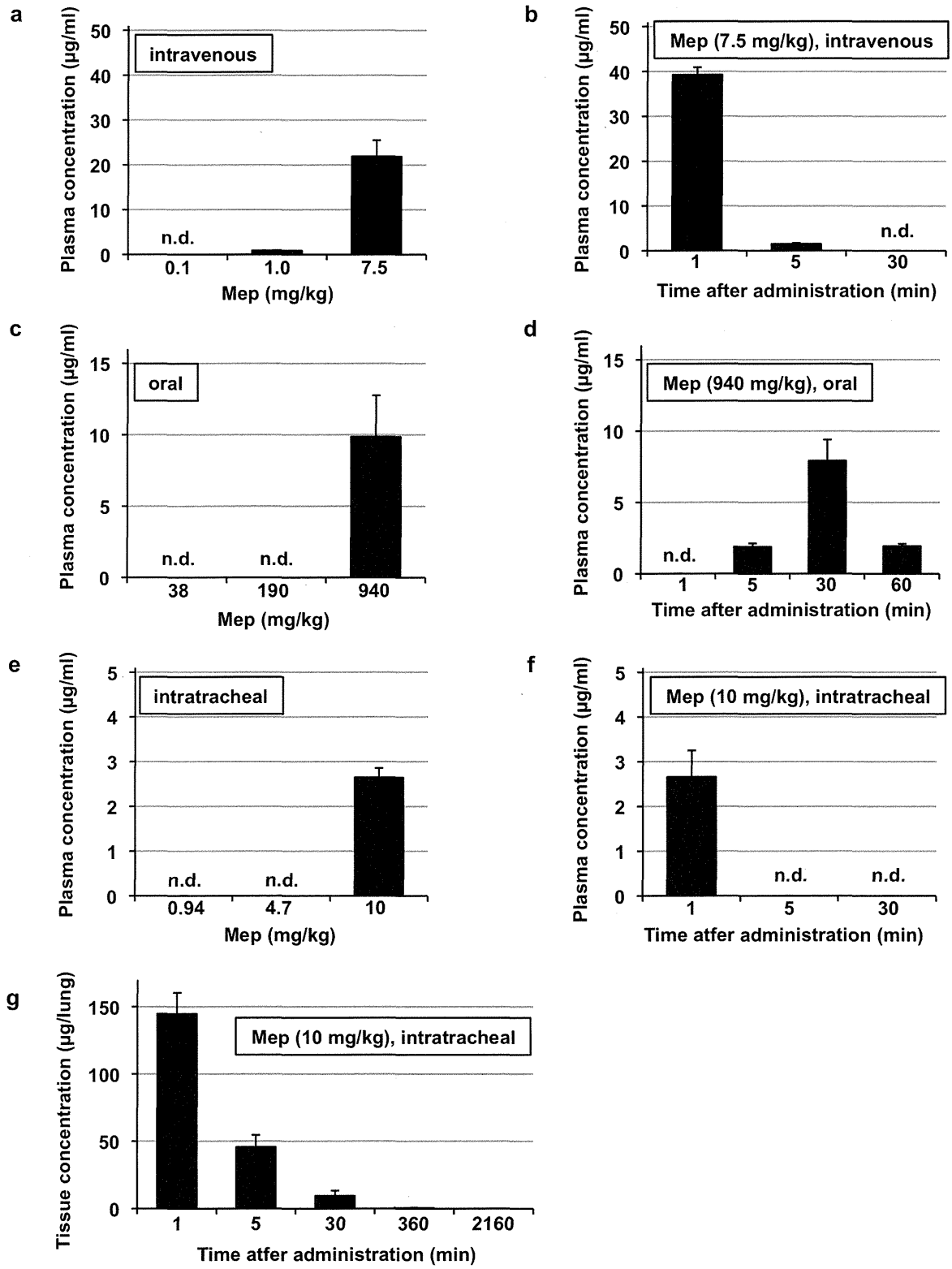

Figure 4 | Determination of the level of mepenzolate after administration through various routes. Mice were administered indicated doses of mepenzolate intravenously (a, b), orally (c, d) or intratracheally (e–g). After indicated periods (b, d, f, g), 1 min (a, e) or 30 min (c), blood samples (a–f) or lung homogenates (g) were prepared and the level of mepenzolate was determined as described in the Materials and Methods. Values are mean ± S.E.M. (n = 3–4). n.d., not detected.

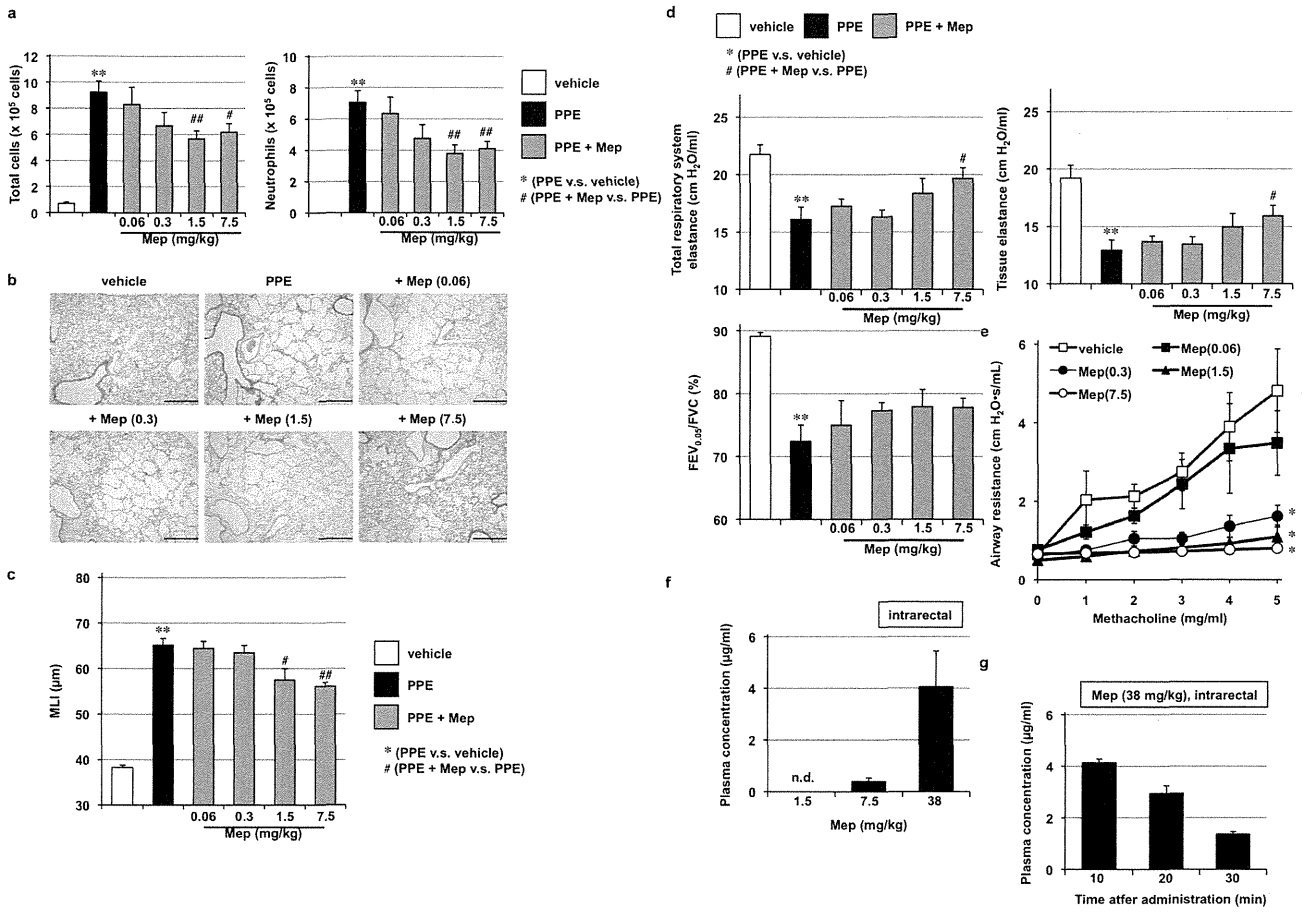

Figure 5 | Effect of intrarectal administration of mepenzolate on PPE-induced pulmonary damage and methacholine-induced airway constriction. Administration of PPE, mepenzolate and methacholine was done, as described in the legend of Fig. 1, except that mepenzolate was administered intrarectally (a–e). Analysis of inflammatory responses (a), histopathological examination (scale bar, 500 μm) (b), determination of the MLI (c), measurement of lung mechanics and respiratory function (d) and measurement of airway resistance (e) were carried out as described in the legend of Fig. 1. Mice were administered indicated doses of mepenzolate intrarectally. After 10 min (f) or indicated periods (g), blood samples were taken and the plasma level of mepenzolate was monitored as described in the legend of Fig. 4. Values represent mean ± S.E.M. ($n = 4$–12). * or $^{\#}$ $P < 0.05$; ** or $^{\#\#}$ $P < 0.01$; n.d., not detected.

the effective dose of mepenzolate was decreased. However, as for the oral and intravenous routes of administration, intrarectally administered mepenzolate exerted both beneficial and adverse side-effects at roughly similar doses.

To determine the appropriate administration route of candidate drugs in a clinical setting, the most important factor is the balance between efficacy and safety. To estimate this factor in animals, the ratio between doses showing adverse effects and efficacy is useful. We calculated this index (Table 1) and results show the superiority of the pulmonary administration route for mepenzolate compared to other routes. The quality of life (QOL) of patients is also an important factor, for which the intravenous route of administration has a disadvantage. As well as oral administration, pulmonary administration (such as inhalation) would not overly affect the QOL of COPD patients given that most of these patients would already be required to take bronchodilators and/or steroids on a daily basis at home through inhalation.

On the other hand, one of the main advantages of the oral route of mepenzolate administration is that it already has regulatory approval, and most pre-clinical tests (such as toxicity and pharmacokinetic tests) could be omitted if the dose for a new indication (COPD) is less than that for the approved indication (gastrointestinal disorders). However, we found that the dose of orally administered drug required to protect against PPE-induced pulmonary damage was much higher than that at which fecal pellet output is affected, suggesting that the clinical dose of mepenzolate for the treatment of COPD would be higher than the already approved dosage. On the other hand, if mepenzolate is developed as a drug to be administered via the pulmonary route, although some pre-clinical tests (such as toxicity and pharmacokinetic tests) are required, other tests (such as genotoxicity tests) could be omitted. Furthermore, because the dose required to protect against PPE-induced pulmonary damage via the intratracheal route was much lower than the orally administered dose that affects fecal pellet output, it could be postulated that the clinical dose of mepenzolate required for the treatment of COPD patients may be lower than the already approved dose if this drug is developed as a drug to be administered intrapulmonary. This could decrease the risk of adverse effects in a clinical setting. In conclusion, we propose that the pulmonary administration of mepenzolate may be superior to other administration routes for the treatment of COPD.

Methods

Chemicals and animals. Mepenzolate, PPE and HPLC-grade acetonitrile were obtained from Sigma-Aldrich (St. Louis, MO). Novo-Heparin for injection was from Mochida Pharmaceutical Co. (Tokyo, Japan). Chloral hydrate was from Nacalai Tesque (Kyoto, Japan). Diff-Quik was from the Sysmex Co (Kobe, Japan). Sodium 1-propanesulfonate was from Tokyo Kasei Chemical Co (Tokyo, Japan). The Amicon utra-0.5 centrifugal filter unit was purchased from Merck Millipore (Billerica, MA).

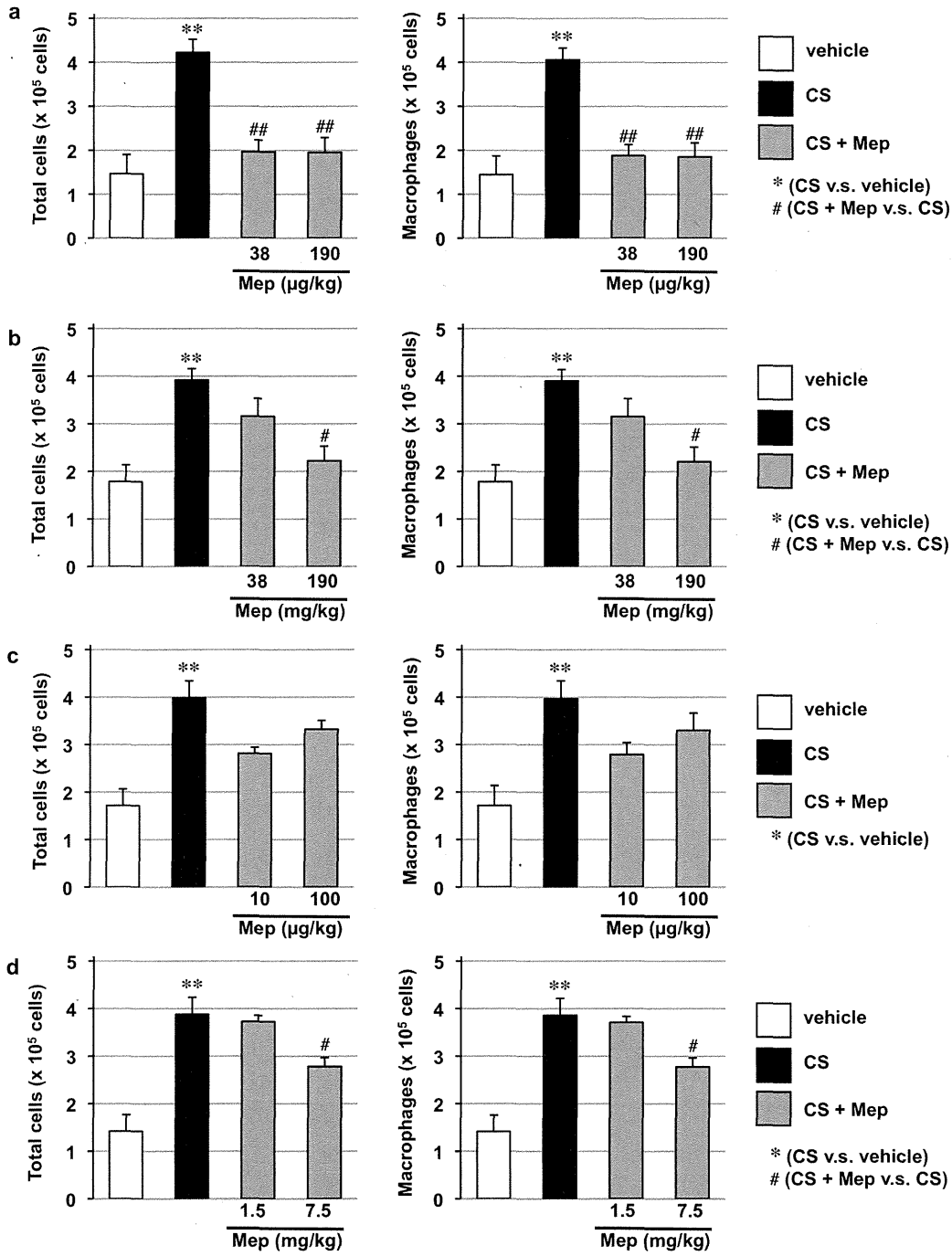

Figure 6 | Effect of mepenzolate on CS-induced pulmonary inflammatory responses. Mice were exposed to CS (3 times/day) and intratracheally (a), orally (b), intravenously (c) or intrarectally (d) administered indicated dose of mepenzolate (once daily) for 3 days as described in the Materials and Methods. Six hours after the last CS exposure, BALF was prepared and the total cell number and the number of macrophages were determined as described in the Materials and Methods. Values represent mean ± S.E.M. (n = 4–8). * or $^{\#}$ $P < 0.05$; ** or $^{\#\#}$ $P < 0.01$.

Formalin neutral buffer solution, potassium dihydrogen phosphate and methylcellulose were from WAKO Pure Chemicals (Tokyo, Japan). Mayer's hematoxylin, 1% eosin alcohol solution and malinol were from MUTO Pure Chemicals (Tokyo, Japan). ICR mice (4–6 weeks old, male) were purchased from Charles River (Yokohama, Japan). The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committee of Keio University.

Treatment of mice with PPE, CS and drugs. Mice maintained under anesthesia with chloral hydrate (500 mg/kg) were given one intratracheal administration of PPE (15 U/kg) and mepenzolate (various doses) in PBS (1 ml/kg) via micropipette. For control mice, PBS alone was administered by the same procedure.

ICR mice were exposed to CS by placing 15–20 mice in a chamber (volume, 45 L) connected to a CS-producing apparatus. Commercial non-filtered cigarettes (Peace®; Japan Tobacco Inc., Tokyo, Japan) that yielded 28 mg tar and 2.3 mg nicotine on a standard smoking regimen were used. Mice were exposed to the smoke of 2 cigarettes for 20 min, 3 times/day for 3 days. The apparatus was configured such that each cigarette was puffed 15 times over a 5 min period.

For the oral or intrarectal mode of administration, mepenzolate (various doses) in 1% methylcellulose was administered by sonde. For control mice, 1% methylcellulose alone was administered by the same procedure.

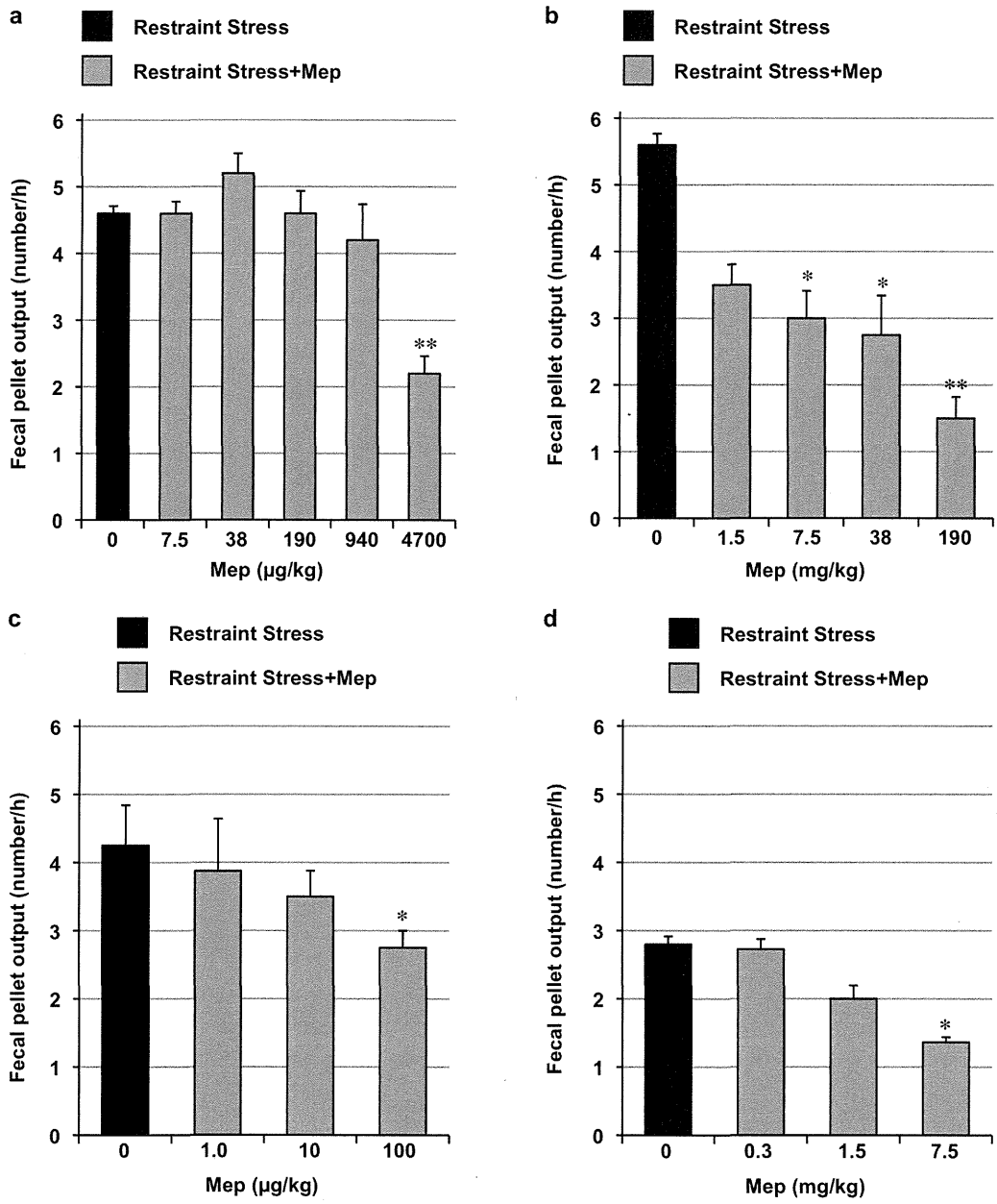

Figure 7 | Effect of mepenzolate on fecal pellet output. Mice were administered indicated doses of mepenzolate intratracheally (a), orally (b), intravenously (c) or intrarectally (d). One hour later, mice were exposed to restraint stress. The number of fecal pellets excreted during the restraint stress period (1 h) was determined. Values represent mean ± S.E.M. (n = 4–15). * $P < 0.05$; ** $P < 0.01$.

For the intravenous administration of mepenzolate, mice were maintained under anesthesia with chloral hydrate (500 mg/kg) and mepenzolate (various doses) in PBS was administered by syringe via a 26 G needle (TERUMO, Tokyo, Japan). For control mice, PBS alone was administered by the same procedure.

At day 0, the administration of mepenzolate was performed 1 h (intratracheal administration) or 0.5 h (other routes of administration) prior to the PPE administration or the CS exposure.

Preparation of BALF and cell count method. BALF was collected by cannulating the trachea and lavaging the lung with 1 ml of sterile PBS containing 50 U/ml heparin (2 times). About 1.8 ml of BALF was routinely recovered from each animal. The total cell number was counted using a hemocytometer. Cells were stained with Diff-Quik reagents after centrifugation with Cytospin® 4 (Thermo Electron Corporation, Waltham, MA), and the ratio of number of neutrophils to total cell number was examined to determine the number of neutrophils.

Histopathological analysis. Lung tissue samples were fixed in 10% formalin neutral buffer solution for 24 h at a pressure of 25 cmH_2O, and then embedded in paraffin before being cut into 4 μm-thick sections. Sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution (H & E staining). Samples were mounted with malinol and inspected with the aid of an Olympus BX51 microscope (Tokyo, Japan).

To determine the MLI (an indicator of airspace enlargement), 20 lines (500 μm) were drawn randomly on the image of a section and intersection points with alveolar walls were counted to determine the MLI. This morphometric analysis was conducted by an investigator blinded to the study protocol.

Measurement of lung mechanics, airway resistance and $FEV_{0.05}$/FVC. Lung mechanics and airway resistance were monitored with a computer-controlled small-animal ventilator (FlexiVent, SCIREQ, Montreal, Canada), as described previously[18,19]. Mice were anesthetized with chloral hydrate (500 mg/kg), a tracheotomy was performed, and an 8 mm section of metallic tube was inserted into the trachea. Mice were mechanically ventilated at a rate of 150 breaths/min, using a tidal volume of 8.7 ml/kg and a positive end-expiratory pressure of 2–3 cmH_2O.

Total respiratory system elastance and tissue elastance were measured by the snapshot and forced oscillation techniques, respectively. All data were analysed using FlexiVent software (version 5.3; SCIREQ, Montreal, Canada).

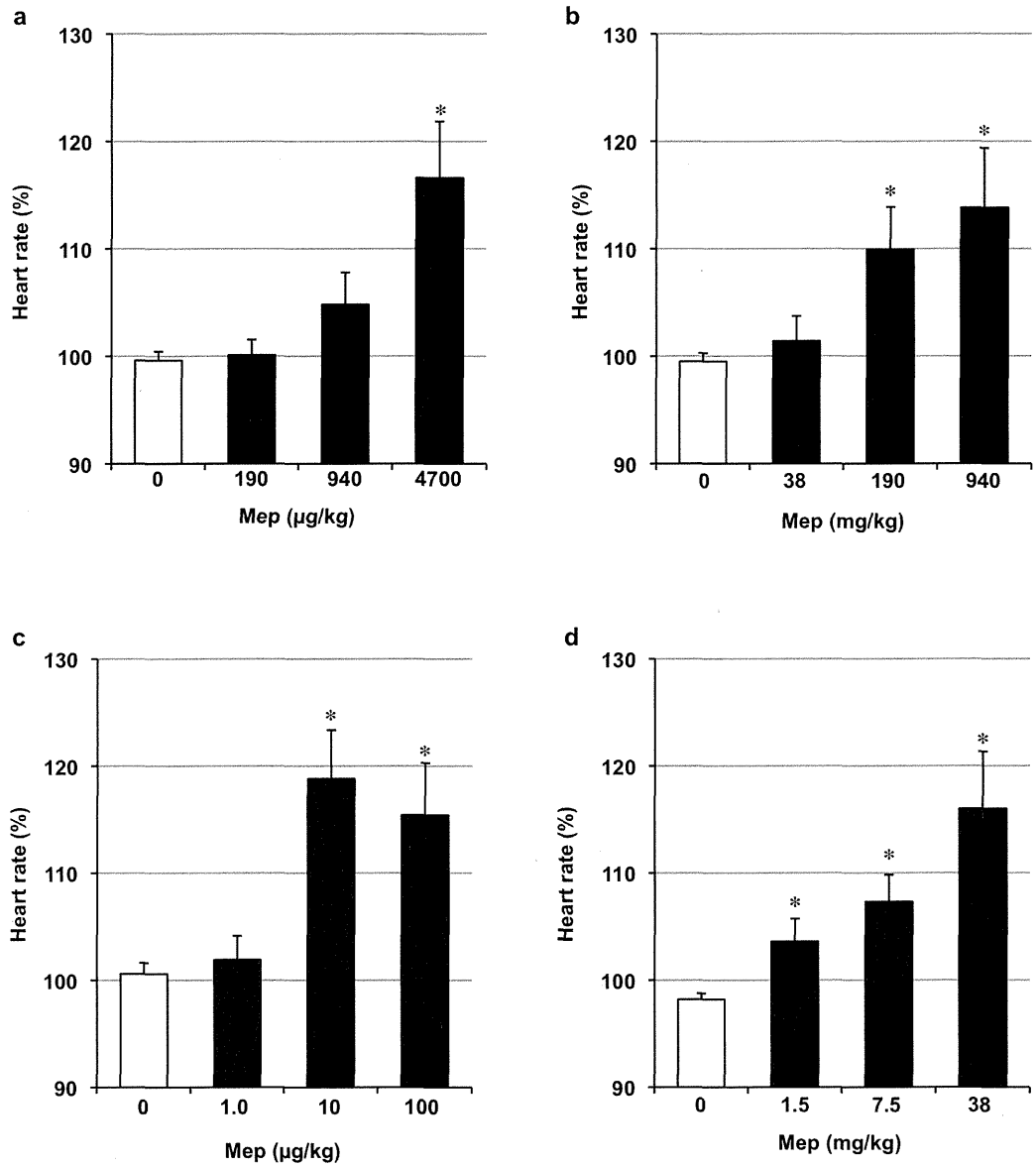

Figure 8 | Effect of mepenzolate on heart rate. Mice were administered indicated doses of mepenzolate intratracheally (a), orally (b), intravenously (c) or intrarectally (d). The alteration of heart rate (beats per minute) by the mepenzolate administration was monitored as described in the Materials and Methods. Mepenzolate-dependent alteration of heart rate from the baseline to the peak is shown. Values represent mean ± S.E.M. ($n = 3$–7). * $P < 0.05$; ** $P < 0.01$.

For measurement of methacholine-induced increases in airway resistance, mice were exposed to nebulized methacholine (1 mg/ml) five times for 20 sec with a 40 sec interval, and airway resistance was measured after each methacholine challenge by the snapshot technique. All data were analysed using the FlexiVent software.

Determination of the FEV0.05/FVC (forced expiratory volume in the first 0.05 seconds to forced vital capacity) ratio was performed with the same computer-controlled small-animal ventilator connected to a negative pressure reservoir (SCIREQ, Montreal, Canada), as described previously[18,19]. Mice were tracheotomised and ventilated as described above. The lung was inflated to 30 cmH_2O over one second and held at this pressure. After 0.2 sec, the pinch valve (connected to ventilator) was closed and after 0.3 sec, the shutter valve (connected to negative pressure reservoir) was opened for exposure of the lung to the negative pressure. The negative pressure was held for 1.5 sec to ensure complete expiration. FEV0.05/FVC was determined using the FlexiVent software.

Table 1 | Efficacy versus toxicity ratio for different routes of mepenzolate administration

Administration route	Intratracheal	Oral	Intravenous	Intrarectal
Efficacy	38 μg/kg	190 mg/kg	10 μg/kg	1.5 mg/kg
Toxicity	4700 μg/kg	7.5 mg/kg	10 μg/kg	1.5 mg/kg
Toxicity/Efficacy	120	0.04	1	1

The effective dose (efficacy) was determined as the minimum dose required to significantly suppress the PPE-induced increase in MLI (Figs. 1c, 2c, 3c and 5c). The toxic dose (toxicity) was determined as the minimum dose required to significantly affect either fecal pellet output or heart rate (Figs. 7 and 8). The ratio of the toxic dose versus the effective dose for each route of administration is shown.

Analysis of fecal pellet output. Mice were subjected to restraint stress by being placed individually into a 50 ml tube (Becton Dickinson, Franklin Lakes, NJ) for 1 h, as described previously[28]. These tubes are small enough to restrain a mouse so that it is able to breathe but unable to move freely. The number of fecal pellets excreted during the restraint stress period (1 h) was measured.

Measurement of heart rate. Heart rate was measured with a MouseOx system (STARR Life Sciences Corp., Allison Park, PA), as described previously[29]. Mice were anesthetized with chloral hydrate (500 mg/kg) and the sensor was attached to the thigh. Heart rate was determined using MouseOx software (STARR Life Sciences Corp., Allison Park, PA).

Determination of the level of mepenzolate in vivo. After administration of mepenzolate, blood samples (800 μl) were taken periodically into centrifuge tubes containing heparin (50 μl) and centrifuged immediately (1000 × g, 10 min) to obtain the sample. Whole lungs were taken from mepenzolate-treated mice, homogenised in sterile PBS containing 50 U/ml heparin, and centrifuged (14, 000 × g, 1 min) to obtain the sample. An aliquot (300 μl) of each sample was ultrafiltered with an Amicon utra-0.5 centrifugal filter to extract the mepenzolate. The filtrate was analysed by analytical HPLC with a reverse-phase column (TSKgel Super-ODS, 150 × 4.6 mm, 2 μm, Tosoh Co., Tokyo, Japan), Waters 2695 Alliance separation module, and a Waters 2996 photodiode array detector (Waters, Milford, MA). Solution containing 30% (v/v) acetonitrile and 14 mM potassium dihydrogen phosphate/sodium 1-propanesulfonate buffer was used at a flow rate of 0.3 ml/min. Detection was performed at an optical density of 220 nm.

Statistical analysis. All values are expressed as the mean ± S.E.M. Two-way ANOVA followed by the Tukey test or the Student's *t*-test for unpaired results was used to evaluate differences between three or more groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$.

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Additional information

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Mechanisms of *Helicobacter pylori* antibiotic resistance and molecular testing

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Antibiotic resistance in *Helicobacter pylori* (*H.*pylori) is the main factor affecting the efficacy of current treatment methods against infection caused by this organism. The traditional culture methods for testing bacterial susceptibility to antibiotics are expensive and require 10–14 days. Since resistance to clarithromycin, fluoroquinolone, and tetracycline seems to be exclusively caused by specific mutations in a small region of the responsible gene, molecular methods offer an attractive alternative to the above-mentioned techniques. The technique of polymerase chain reaction (PCR) is an accurate and rapid method for the detection of mutations that confer antibiotic resistance. This review highlights the mechanisms of antibiotic resistance in *H. pylori* and the molecular methods for antibiotic susceptibility testing.

Keywords: *Helicobacter pylori*, 23S rRNA, gyrA, PBP1, 16S rRNA

INTRODUCTION

H. pylori is identified as a Group 1 carcinogen by the World Health Organization International Agency for Research on Cancer (WHO/IARC), and is associated with the development of gastric cancer. Eradication of *H. pylori* infection has been reported as an effective strategy in the treatment of peptic ulcers and gastric mucosa-associated lymphoid tissue lymphoma as well as in the prevention of gastric cancer (Fukase et al., 2008).

Triple treatment including proton pump inhibitor-amoxicillin and clarithromycin or metronidazole proposed at the first Maastricht conference is globally accepted as the technique used to treat *H. pylori* infection. However, recent data show that efficacy of this combination has decreased, with successful cure in only 70% patients (Nishizawa et al., 2014). The 4th edition of the Maastricht consensus recommended a threshold of 15–20% to separate regions of high and low clarithromycin resistance (Malfertheiner et al., 2012). In areas of low clarithromycin resistance, treatments containing clarithromycin are recommended as a first-line empirical treatment. After failure of clarithromycin-containing therapy, either bismuth-containing quadruple therapy or levofloxacin-containing triple therapy is recommended. In areas of high clarithromycin resistance, bismuth-containing quadruple treatments (bismuth subsalicylate, PPI, tetracycline, and metronidazole) are recommended as a first-line empirical treatment. After failure of the quadruple therapy, levofloxacin-containing triple therapy is recommended. After failure of the second line of treatment, subsequent treatment methods should be guided by antimicrobial susceptibility testing whenever possible. The alternative candidates for third-line therapy are rifabutin, fluoroquinolones, tetracycline, furazolidone, and high-dose PPI/amoxicillin therapy (Nishizawa et al., 2008, 2012; Zhang et al., 2014). The traditional culture test for bacterial susceptibility to antibiotics is requires 10–14 days, and is not routinely performed in clinical practice. Minimal inhibitor concentration (MIC)-based individualized treatment for *H. pylori* infection is not prevalent among general practitioners. Molecular techniques for antibiotic susceptibility testing can determine bacterial susceptibility to some antibiotics within a few days. We reviewed the mechanisms of antibiotic resistance in *H. pylori* and the molecular techniques for antibiotic susceptibility testing.

CLARITHROMYCIN

Clarithromycin resistance possibly results from the use of the antibiotic in the pediatric, respiratory, and otorhinolaryngology fields (Kaneko et al., 2004). Global clarithromycin-resistance rates have increased from 9% in 1998 to 17.6% in 2008 in Europe and from 7% in 2000 to 27.7% in 2006 in Japan (Asaka et al., 2010). In patients with clarithromycin-resistant *H. pylori*, it has been reported that the eradication rate achieved with clarithromycin-based regimens shows a marked decrease (Nishizawa et al., 2014). Therefore, the choice of clarithromycin after drug susceptibility testing is promising approach.

The bacteriostatic activity of macrolides such as erythromycin and clarithromycin depends on the capacity to inhibit protein

synthesis by binding to the 23S ribosomal subunit (23S rRNA). Extensive studies have demonstrated that point mutations in the peptidyltransferase region encoded in domain V of 23S rRNA are responsible for macrolide resistance. These mutations result in the inhibition of binding between clarithromycin and the ribosomal subunit dedicated to specific antibiotic-related protein synthesis (Versalovic et al., 1996). In particular, the main *23S rRNA* mutations include an adenine-to-guanine transition at positions 2142 (11.7%) and 2143 (69.8%), and an adenine-to-cytosine transversion at position 2142 (2.6%).

Several other point mutations have been identified, such as A2115G, T2117C, G2141A, T2182C, G2224A, C2245T, T2289C, C2611A, and T2717C (Francesco et al., 2011). Besides their low frequency, the clinical relevance of A2115G, T2117C, G2141A, G2224A, T2289C, C2245T mutations is still not proven. The T2182C, C2611A, and T2717C have been associated with low resistance levels (Francesco et al., 2011).

Another relevant mechanism for macrolide resistance is attributed to the efflux pump system. At least 4 conserved families of efflux systems associated with bacterial resistance to antibiotics have been identified (Bina et al., 2000). One of these, widespread among gram-negative bacteria, is the resistance-nodulation-cell division (RND) family of efflux systems (Ma et al., 1995). Bina et al. identified the RND efflux system in *H. pylori*. *HP0605* from *H. pylori* is a homolog of the *E. coli* gene (*TolC*) encoding the outer membrane protein, TolC, while *HP0607* and *HP0606* and are homologs of the *E. coli* genes *acrA* and *acrB* encoding the membrane fusion and RND cytoplasmic pump protein, respectively (Bina et al., 2000). Kutschke et al. reported that *HP0607* knockout mutants exhibited increased susceptibility to penicillin G, cefotaxime, erythromycin, clarithromycin, tetracycline, clindamycin, novobiocin, and ethidium bromide (Kutschke and de Jonge, 2005). We previously investigated the efficacy of efflux pump inhibitor (Phe-Arg-beta-naphthylamide) in 15 clarithromycin resistant *H. pylori*. In all 15 strains, efflux pump mRNA was expressed, and the MIC of clarithromycin were decreased by using efflux pump inhibitor, despite possessing *23s rRNA* point mutations. In addition, the MIC of clarithromycin was decreased by the efflux pump inhibitor in a concentration-dependent fashion (Hirata et al., 2010).

Most clarithromycin-resistant strains of *H. pylori* have a A2142G or A2143G mutation. Furuta et al. designed the forward primer FP2143G and the reverse primer RP2142G, which specifically anneal to A2143G and A2142G mutated sequences of the *H. pylori 23S rRNA* gene (Furuta et al., 2007). *H. pylori* strains with A2142G, A2143G, and wild-type genotype can be differentiated by amplicon size using the allele-specific, primer-polymerase chain reaction (PCR) method, which is useful and requires only a single PCR run.

Wang et al. reported multiplex sequence analysis (Wang et al., 1999), wherein positions 2142 and 2143 have an AA sequence in wild-type cells, while mutant cells show a change to GA (A2142G) and AG (A2143G), respectively. In the presence of dCTP, dATP, and ddGTP terminating DNA strand elongation results in products of unique length, depending on type of mutation.

Dual priming oligonucleotide (DPO)-PCR is a multiplex PCR assay that increases specificity and sensitivity of detection compared to conventional PCR, blocking non-specific binding sites therefore eliminating imperfect primer annealing. Seeplex® ClaR-*H. pylori* ACE detection (Seegene, Inc., Seoul, Korea) is commercially available DPO-PCR kit to detect *H. pylori* and A2142G, A2143G mutations. Although Seeplex® ClaR-*H. pylori* ACE detection kit does not allow of detection of A2142C mutation, the mutation is less common (<5% of resistant isolates). Lehours et al. reported E-test and DPO-PCR were concordant with regard to clarithromycin susceptibility in 95.3% of the cases (41/43) (Lehours et al., 2011) (**Table 1**).

Versalovic et al. developed a method based on rapid restriction analysis of the amplicon obtained from *H. pylori* (Versalovic et al., 1996). Single point mutations at positions 2143, 2142, and 2717 generate specific restriction sites, namely *Bsa*I, *Mbo*II, and *Hha*I, respectively, which can be used in rapid screening for clarithromycin resistance (Masaoka et al., 2004). Fontana et al. developed a new method involving semi-nested PCR and digestion by *Mbo*II, *Bsa*I, and *Hha*I using stool samples. This method is non-invasive and easy to perform (Fontana et al., 2003).

Klesiewicz et al. evaluated the occurrence of A2143G and A2142G mutations in 21 *H. pylori* strains resistant to clarithromycin. The point mutations were detected by PCR followed by restriction fragment length polymorphism (RFLP) analysis. Nine *H. pylori* strains exhibited A2143G mutation and nine *H. pylori* strains exhibited A2142G mutation. The results of RFLP analysis of 3 clarithromycin-resistant strains were negative for both mutations (Klesiewicz et al., 2014).

Wu et al. evaluated the utility of the string test to detect genotypic clarithromycin-resistant *H. pylori* by PCR-RFLP. In the string test, a 90-cm nylon string coiled inside a gelatin capsule was used. A free-end looped string protrudes through a hole in the other end of the capsule. Before the capsule was swallowed, 10–20 cm of the free-end string was pulled out and its position was ensured by adhesion of a small piece of tape to the patient's cheek. It was swallowed with 300 mL of water after 8 h of fasting. One hour after swallowing, the string was retrieved. Approximately 0.5 mL of gastric juice with *H. pylori* attached by every 10 cm of the string was reasonable for molecular biological analysis. Forty three isolates were successfully cultured in 79 patients in whom *23S rRNA* was successfully amplified. Of 5 patients with clarithromycin-resistant *H. pylori*, *23S rRNA* of *H. pylori* isolates from 4 patients could be digested by *Bsa*I. In 38 susceptible isolates, *23S rRNA* of *H. pylori* isolates from 36 patients could not be digested by either *Bsa*I or *Bbs*I. The sensitivity and specificity of the string test to detect genotypic clarithromycin resistance were 66.7 and 97.3%, respectively (Wu et al., 2014).

The restriction enzyme is capable of identifying an A-to-G mutation by the creation of a restriction site, but if an A-to-C mutation occurs, the restriction enzyme may not restrict the DNA at that site. Stone et al. developed a rapid assay based on PCR followed by oligonucleotide ligation for rapid detection of these point mutations, which could differentiate between *H. pylori* strains with A2142G, A2143G, A2142C, and wild–type genotype (Stone et al., 1997).

Several quantitative PCR assays for the determination of clarithromycin susceptibility in *H. pylori* have subsequently been

Table 1 | Commercially available molecular methods for *H. pylori* antibiotics resistance.

Product name	Seeplex	ClariRes	GENECUBE	HelicoDR	
Manufacturer (Country)	Seegene (Korea)	Ingenetix (Austria)	TOYOBO (Japan)	Hain Life Science (Germany)	
Assay technique	Dual priming oligonucleotide PCR	Real-time PCR using biprobe	Real-time PCR using quenching probe	DNA strip genotyping test combining PCR and hybridization	
Time-to-result	4 h		30 min	6 h	
Target gene Detectable mutation	*23S rRNA* A2142G, A2143G	*23S rRNA* A2142G, A2143G A2142C	*23S rRNA* A2142G, A2143G	*23S rRNA* A2142G, A2143G A2142C	*gyrA* 87 (Asn to Lys) 91 (Asp to Gly, Asn, or Tyr)
H. pylori detection	Sensitivity 97.7% Specificity 83.1% (Reference test: culture)	Sensitivity 100% Specificity 98–100% (Reference test: culture, RUT, histology)	Sensitivity 100% Specificity 100% (Reference test: Hp -IgG, RUT, UBT)		
Comparison with direct sequencing			Sensitivity 100% Specificity 100%	Sensitivity 94.9% Specificity 87.1%	Sensitivity 98.2% Specificity 80.0%
Comparison with susceptibility test	Concordant rate 95.3% (41/43)	Sensitivity 82-100% Specificity 100%		Sensitivity 94% Specificity 99%	Sensitivity 87% Specificity 98.5%

UBT: ^{13}C-urea breath test, Hp-IgG: serum anti-H. pylori -IgG, RUT: rapid urease test.

reported. These include a two-step process involving LightCycler PCR for the detection of *H. pylori* followed by melting curve analysis using probe hybridization to detect resistance (Oleastro et al., 2003).

GENECUBE® (TOYOBO Co., LTD. Japan) is a novel, fully automated rapid genetic analyzer capable of extracting nucleic acids from biological material, preparing reaction mixtures, and amplifying the target gene all within 30 min. The amplified target DNA is hybridized with a fluoresce-labeled oligonucleotide (a Qprobe). Upon binding to the target DNA, the Qprobe fluorescence is quenched by the guanine bases in the target. However, the fluorescence reappears as the Qprobe disassociated from the melting target. By detecting this change in fluorescence intensity, A2143G and A2142G mutations are detected. Furuta et al. reported the GENECUBE® genotyping results of the *23S rRNA* gene from gastric tissue samples ($n = 50$) were in complete agreement with those for direct sequencing. Furthermore, gastric juice samples were collected during gastroduodenoscopy in 132 patients. Twenty six of the 132 samples were *H. pylori*-negative based on analysis of serum anti-*H. pylori*-IgG, urease and the ^{13}C-urea breath test, and the remaining 106 were *H. pylori*-positive. The GENECUBE® could detect *H. pylori* infection in all patients infected with *H. pylori* based on analysis of serum anti-*H. pylori*-IgG, urease and the ^{13}C-urea breath test. Thus, the sensitivity, specificity and validity of the GENECUBE® assay were all 100% (Furuta et al., 2013). GENECUBE® is commercially available, it is not approved for clinical diagnostic use (research use only).

ClariRes® assay (Ingenetix, Vienna, Austria) is a novel commercially available quantitative PCR assay allowing *H. pylori* detection and clarithromycin susceptibility testing in either gastric biopsy or stool specimens. In the biprobe quantitative PCR protocol, followed by hybridization melting point analysis, A2143G, A2142G, and A2142C mutations are detected.

Schabereiter et al. evaluated the clinical usefulness of ClariRes® test in 92 patients who underwent endoscopy. 45 were found to be *H. pylori* infected and invariably were also culture positive. With respect to the detection of *H. pylori* infection, ClariRes® test showed sensitivities of 100% and a specificity of 98%. Of the 45 isolates, 11 were shown to be resistant to clarithromycin by E-test. Compared to E-test, the sensitivity and specificity of ClariRes® test for clarithromycin resistance were 82% and 100% (Schabereiter-Gurtner et al., 2004). Scaletsky et al. evaluated the clinical usefulness of ClariRes® test in Brazilian children. Forty five of the 217 samples were *H. pylori*-positive based on analysis of culture, rapid urease test, or histological examination, and the remaining 172 were *H. pylori*-negative. The sensitivity, specificity and validity of the ClariRes® assay were all 100% for the detection of *H. pylori* infection. In the 45 culture positive patients, the ClariRes® genotyping results of the *23S rRNA* gene from gastric tissue samples were in complete agreement with those for the E-test (Scaletsky et al., 2011).

Can et al. developed a fluorescent *in situ* hybridization (FISH) method to detect *H. pylori* and determine clarithromycin resistance in formalin-fixed, paraffin-embedded, gastric biopsy specimens (Can et al., 2005). Cerqueira et al. evaluated a peptide nucleic acid-FISH method for *H. pylori* clarithromycin resistance detection in paraffin-embedded gastric biopsy specimens. In the retrospective study ($n = 30$ patients), full agreement between peptide nucleic acid-FISH and PCR-sequencing was observed. Compared to the culture followed by E-test, the specificity and sensitivity of peptide nucleic acid-FISH were 90.9 and

84.2%, respectively. In the prospective cohort ($n = 93$ patients), 21 cases were positive by culture. For the patients harboring clarithromycin-resistant *H. pylori*, the method showed sensitivity of 80.0% and specificity of 93.8% (Cerqueira et al., 2013).

Xuan et al. developed an enzymatic colorimetric DNA chip (Xuan et al., 2009) including A2142G, A2142C, A2143G, A2143C, and G2224A mutations, where results were 96.8% (61/63) consistent with those of DNA sequencing. Due to its simplicity and rapidness, the colorimetric DNA chip might be technically feasible for use in general clinical practice.

FLUOROQUINOLONES

The primary resistance of *H. pylori* to fluoroquinolones has been reported to range between 2–22% in different countries or regions (Suzuki et al., 2010). Resistance to fluoroquinolones is easily acquired, and the resistance rate is relatively high in countries with a high consumption of these drugs (Nishizawa et al., 2006).

Fluoroquinolones exert their antimicrobial activity by inhibiting the function of the enzyme DNA gyrase (Moore et al., 1995). The bacterial gyrase is essential for maintaining the DNA helicoidal structure in addition to being involved in DNA replication, recombination and transcription. The gyrase is a tetramer consisting of two A and two B subunits encoded by the *gyrA* and *gyrB* genes, respectively. The A subunit of DNA gyrase is responsible for DNA cleavage and rejoining, is also the site of action of fluoroquinolones (Matsuzaki et al., 2010). Point mutations in the Quinolones Resistance-Determining Region (QRDR) of *gyrA* prevent binding between the antibiotic and the enzyme, conferring antibiotic bacterial resistance (Nishizawa et al., 2009). *H. pylori* does not possess a gene encoding topoisomerase IV, an important fluoroquinolone target in other bacteria. Point mutations in the QRDR of the *gyrA* gene are mainly at amino acid 87 (Asn to Lys or Tyr) or 91 (Asp to Gly, Asn, or Tyr). We previously reported that the MICs of sitafloxacin in *gyrA* mutation-positive strains differed, depending on the position of the *gyrA* mutation (Suzuki et al., 2009a; Matsuzaki et al., 2012). The MICs were higher in N87-mutated strains ($0.21 \pm 0.16\,\mu g/ml$) than in D91-mutated strains ($0.12 \pm 0.11\,\mu g/ml$, $P = 0.03$) (Matsuzaki et al., 2012). Rimbara et al. proposed that mutation at position 463 in *gyrB* would be a novel mechanism of fluoroquinolone resistance in *H. pylori* (Rimbara et al., 2012).

We previously designed the allele-specific primer, which specifically annealed to the C261A, C261G (87 Asn to Lys), G271A (91 Asp to Asn), G271T (91 Asp to Tyr), and A272G (91 Asp to Gly) mutated sequences of the *gyrA* gene in *H. pylori* (Nishizawa et al., 2007). In the allele-specific PCR method, PCR amplification was performed using allele-specific primers in which the second nucleotide from the 3′ end was designed to match the site of the point mutation.

Glocker et al. developed a reliable fluorescence resonance energy transfer-based quantitative PCR method to detect mutations in the *gyrA* gene (Glocker and Kist, 2004). This method was developed on DNA extracts from *H. pylori* isolates from German patients. Because of the known genetic heterogeneity of *H. pylori* (Suerbaum, 2000), the assay may fail with strains isolated outside Germany, but the test could be altered to adapt to the genetic *gyrA* variants found in different geographical regions.

Cambau et al. developed HelicoDR®, a DNA strip genotyping test combining PCR and hybridization (Cambau et al., 2009) that allows the molecular detection of mutations in the *gyrA* gene and 23S rRNA within 6 h. The sensitivity and specificity of detecting resistance using this method were 94 and 99% for clarithromycin and 87% and 98.5% for levofloxacin, respectively. HelicoDR® (Hain Life Science, Germany) is commercially available.

Lee et al. evaluated the clinical usefulness of HelicoDR® test in Korea. Both DNA sequencing after MIC test and HelicoDR® test were performed in *H. pylori* isolates from the gastric mucosa of 101 patients. Among 42 isolates with A2143G mutation by HelicoDR®, 83.3% (35/42) of concordance rate was estimated with direct sequencing method and 85.7% (36/42) for MIC test. Among 43 isolates with amino acid 87 (Asn to Lys) mutation by HelicoDR®, 71.1% (31/43) of concordance rate was estimated with direct sequencing and 88.4% (38/43) for MIC test. Compared to direct sequencing, the sensitivity and specificity of HelicoDR® test for *23S rRNA* mutation were 94.9 and 87.1%, and those for *gyrA* 98.2 and 80.0%. Compared to MIC test, the sensitivity and specificity of HelicoDR® test for clarithromycin resistance were 55.0 and 80.0% and those for fluoroquinolone were 74.4 and 70.0% (Lee et al., 2004).

METRONIDAZOLE

The prevalence of resistance to metronidazole in *H. pylori* has been reported to range between 8 and 80% in different countries (Suzuki et al., 2010). Metronidazole resistance is much higher in developing countries (more than 60%) than in developed countries (Banatvala et al., 1994). Metronidazole is a prodrug that needs to be activated by reduction of the nitro group attached to the imidazole ring. This reduction step leads to the production of DNA-damaging nitroso- and hydroxylamine-containing compounds. The reduction of metronidazole is mainly mediated by oxygen-insensitive NADPH nitroreductase (RdxA), NADPH-flavin-oxidoreductase (FrxA), and ferredoxin-like enzymes (FrxB) in *H. pylori* (Francesco et al., 2011). Different mutations involving the *rdxA* gene have been identified in metronidazole-resistant strains (Masaoka et al., 2006). In the *rdxA* gene, complex genetic events (insertions and deletions of transposons, and missense and frameshift mutations) could be simultaneously present. These mutations are recognized as the main mechanism conferring metronidazole resistance in *H. pylori*. Point mutations in *frxA* and *frxB* can increase bacterial resistance exclusively in the presence of mutations in the *rdxA* gene. We previously demonstrated a novel mechanism of metronidazole resistance in *H. pylori*, namely, aberrant increase of superoxide dismutase expression resulting from the mutation of the ferric uptake regulator (Fur) (Tsugawa et al., 2011). Superoxide dismutase is essential for protection against superoxide attack. Superoxide dismutase is derepressed by mutant-type Fur, which is associated with the development of metronidazole resistance.

Due to various mutations of *rdxA*, molecular antibiotic susceptibility testing is not applicable for metronidazole.

AMOXICILLIN

The prevalence of resistance to amoxicillin has fortunately remained low, where most studies have reported it as less than 2% in all countries, except in Bangladesh (6.6%) (Nahar et al., 2004). Amoxicillin acts by interfering with peptidoglycan synthesis, especially by blocking transporters, namely penicillin binding proteins (PBP). Multiple mutations in *pbp1* gene are the major reason for amoxicillin resistance. Although amoxicillin resistance in *H. pylori* is rare, we previously reported that the MIC_{90} of amoxicillin showed a 2-fold increase with the failure of each eradication treatment (Nishizawa et al., 2011a). Low-level resistant strains (MIC: 0.06–0.25 μg/ml) had 0–2 substitutions, while high-level resistant strains had 1–3 substitutions. Low-level resistance to amoxicillin is linked to a point mutation on *pbp1*, and the accumulation of *PBP1* mutations could result in a gradual increase in amoxicillin resistance. Although production of β-lactamase is rare and almost inactive in *H. pylori*, Tseng et al. reported that high-level amoxicillin resistance is associated with β-lactamase production in *H. pylori* (Tseng et al., 2009). Due to different mutations of *PBP1*, molecular antibiotic susceptibility testing is not applicable for amoxicillin.

TETRACYCLINE

The prevalence of resistance to tetracycline also has fortunately remained low; it was reported to be less than 2% in most studies (Suzuki et al., 2010). Tetracyclines are bacteriostatic drugs that exert their antimicrobial effect on the 30S subunit of the ribosome and block the binding of aminoacyl-tRNA, resulting in impaired protein biosynthesis. The resistance of *H. pylori* to tetracyclines is reported to be caused by mutations in the *16S rRNA* (Gerrits et al., 2002). Simultaneous triple point-mutations at positions 965–967 are recognized to be major responsible for tetracycline resistance. Levels of resistance are proportional to the number of changes in the AGA 965–967. Single and double point-mutations are associated with low and intermediate MIC values, respectively. High resistance levels are observed in the substitution of an AGA with a TTC triplet.

Ribeiro et al. developed a PCR-RFLP assay allowing rapid and reproducible identification of mutations mediating high-level tetracycline resistance in *H. pylori* (Ribeiro et al., 2004). The substitution of an AGA with a TTC triplet creates an additional *Hinf*I restriction site. This PCR-RFLP assay distinguishes high-level tetracycline resistant isolates from low-level tetracycline resistant and tetracycline susceptible *H. pylori* strains.

Glocker et al. developed real-time PCR to detect *16S rRNA* gene mutations that was capable of differentiating between wild-type strains and resistant strains exhibiting single-, double-, or triple-base-pair mutations (Glocker et al., 2005). Future studies need to address the question of whether additional mutations play a role in the resistance of *H. pylori* to tetracycline.

RIFABUTIN

The mean *H. pylori* rifabutin-resistance rate (calculated from 11 studies including 2982 patients) was 1.3% (Gisbert and Calvet, 2012). When only studies including patients naïve to *H. pylori* eradication treatment were considered, this figure was even lower (0.6%). We previously investigated the resistance to rifabutin of *H. pylori* isolated from both general hospital and a hospital specialized for chronic respiratory disease, including pulmonary tuberculosis. Among 94 strains tested, 7 (7.4%) were isolated from patients with a past rifampicin treatment. All these 7 strains showed high rifabutin resistance (Suzuki et al., 2009b). Rifabutin is an antituberculous agent derived from rifamycin-S, which is structurally similar to rifampicin. Rifabutin inhibits the expression of beta-subunit of DNA-dependent RNA polymerase of *H. pylori*, which is encoded by the *rpoB* gene (Nishizawa et al., 2011b). Rifabutin-resistant isolates of *H. pylori* showed mutations in codon 149, codons 525 to 545, or codon 586 (Heep et al., 2002). Due to the different mutations of *rpoB*, the molecular antibiotic susceptibility testing is not applicable for rifabutin.

CONCLUSION

Conventional methods used to assess the level of antibiotic-resistance of *H. pylori* are culture-based methods used in combination with agar dilution or the E-test. However, because of the slow growth and particular requirements of *H. pylori* culture, this approach is not reliable for use in most routine clinical laboratory. Since resistance to clarithromycin, fluoroquinolone, and tetracycline seems to be a result of specific mutations in a small region of the responsible gene, molecular methods offer an attractive alternative. Some reliable molecular methods are commercially available. However, large-scale prospective studies should be performed to assess the full clinical potential of these molecular methods and its economic feasibility. Users should keep in mind that whenever possible *H. pylori* culture should be performed and only in cases where standard microbiology fails, the use of molecular methods are really indicated.

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MicroRNAs in Barrett's esophagus: future prospects

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Esophageal adenocarcinoma is an aggressive malignancy with a poor prognosis. In Western countries, the incidence of esophageal adenocarcinoma has increased dramatically in the last three decades. To improve patient survival and reduce disease burden, early-stage detection, or better yet, preventing the progression of esophageal adenocarcinoma from its premalignant lesions, constitute the best short-term options. Barrett's esophagus is histologically characterized by the replacement of the normal stratified squamous epithelium of the esophagus with a columnar epithelium with intestinal differentiation (Matsuzaki et al., 2010, 2011). Barrett's esophagus is considered to be a complication of gastroesophageal reflux disease and a precursor lesion of esophageal adenocarcinoma. It is generally believed that the progression of Barrett's esophagus involves a series of histological changes: non-dysplastic Barrett's metaplasia, low-grade dysplasia, high-grade dysplasia, and ultimately, adenocarcinoma. Although these features justify endoscopic surveillance for the premalignant stages, patients with Barrett's esophagus show an absolute annual risk of only 0.12% for the development of esophageal adenocarcinoma (Hvid-Jensen et al., 2011). Therefore, recommending the invasive and expensive conventional endoscopic screening procedure is deemed controversial. In fact, Corley et al. reported that, within a large community-based population, endoscopic surveillance of Barrett's esophagus was not associated with a substantial decrease in the risk of death from esophageal adenocarcinoma (Corley et al., 2013). Thus, identification of better risk stratification biomarkers to determine the risk of progression from Barrett's esophagus to esophageal adenocarcinoma may improve disease outcome and make patient management more cost-efficient.

MicroRNAs (miRNAs) are a class of small non-coding endogenous RNAs, 18–25 nucleotides in length, and are capable of simultaneous regulation of genes by binding to target mRNAs, resulting in mRNA degradation or translational inhibition. miRNAs participate in many essential biological processes, including proliferation, differentiation, apoptosis, necrosis, autophagy, and stress responses (Saito et al., 2011b, 2012a). miRNAs have also been shown to play a potential role in cancer pathogenesis through their functions as oncogenes or tumor suppressors, depending on their gene targets (Saito et al., 2009a, 2011a; Nishizawa and Suzuki, 2013). Compared to mRNAs, miRNAs are less numerous in humans and have been proposed to act as better biomarkers by virtue of their small size, greater stability, and capability of regulating hundreds of mRNAs. Therefore, miRNAs profiling could improve the risk stratification for the progression of Barrett's esophagus to esophageal adenocarcinoma.

MiRNAs can be profiled on a genome-wide scale using array or sequencing technologies. However, very few studies have been conducted to identify miRNAs as prognostic biomarkers for the progression of Barrett's esophagus to adenocarcinoma. Although several cross-sectional studies using comprehensive array analysis have been reported (Feber et al., 2008; Kan et al., 2009; Yang et al., 2009; Fassan et al., 2011; Leidner et al., 2012; Wu et al., 2013), their results have proved controversial. They compared the expression of miRNAs across different types of histological specimens such as Barrett's esophagus, low-grade dysplasia, high-grade dysplasia, and esophageal adenocarcinoma, and reported that a substantial number of miRNAs show differential expression in esophageal tissues (Sakai et al., 2013). Indeed, they might be useful in revealing certain mechanisms underlying carcinogenesis. But, they might be difficult to identify risk stratification biomarkers. We should think about much better research strategies.

Recently, two nice studies were reported to identify risk stratification biomarkers for Barrett's esophagus: one prospective study and one cross-sectional study. First, Revilla-Nuin et al. have reported a set of miRNAs associated with this progression and provided further validation in two groups of patients with Barrett's esophagus, who either developed or did not develop adenocarcinoma, over a course of 5 years (Revilla-Nuin et al., 2013). Among 24 patients with Barrett's esophagus, 7 patients progressed to adenocarcinoma while the other 17 did not. Four miRNAs (*miR-192*, *miR-194*, *miR-196a*, and *miR-196b*) were found to show significantly higher expression in patients with progression to esophageal adenocarcinoma than in patients who did not show disease progression. Second, Saad et al. conducted a notable comprehensive microarray profiling for identifying the specific miRNA signature associated with esophageal adenocarcinoma (Saad et al., 2013). They analyzed 13 samples from isolated Barrett's esophagus, 10 from Barrett's esophagus adjacent to high-grade dysplasia, 17 from high-grade dysplasia, and 34 from esophageal adenocarcinoma tissue. They

identified that *miR-21*, *miR-31*, *miR-192*, and *miR-194* were upregulated in Barrett's esophagus adjacent to high-grade dysplasia lesions as compared to isolated Barrett' esophagus. In addition, these 4 miRNAs were upregulated in a progressive manner through the Barrett's metaplasia-dysplasia-adenocarcinoma sequence. More importantly, this study provided findings for Barrett's esophagus for two groups: isolated Barrett's esophagus vs. Barrett's esophagus adjacent to high-grade dysplasia. The limitations of both two papers include the very small sample size. Larger prospective multi-institutional studies are warranted to confirm this result. Another criticism against the studies using comprehensive microarray analysis is that these could not provide the insights how miRNAs may exert their effects (Saito et al., 2009b, 2012b, 2013).

Since clinical predictors of increased risk of esophageal adenocarcinoma, namely, the length of Barrett's esophagus, male gender, older age, current tobacco smoking, alcohol consumption, central obesity, and bile reflux, have been established, the association between the expression levels of miRNA in Barrett's esophagus and these clinical risk factors would require further investigation. We had recently reported that expression levels of *miR-221* and *miR-222* increased when cultured esophageal epithelial cells were exposed to bile acids. *miR-221* and *miR-222* are known to specifically target p27Kip1, which in turn inhibits the proteasomal protein degradation of CDX2 (caudal-related homolog 2) (Matsuzaki et al., 2013). Furthermore, *miR-221* and *miR-222* expressions are higher in esophageal adenocarcinoma than in the surrounding Barrett's esophagus. We also confirmed that the levels of p27Kip1 and CDX2 were lower in areas of esophageal adenocarcinoma than in those of Barrett's esophagus. Thus, we showed that the degradation of CDX2 was enhanced by upregulation of *miR-221* and *miR-222* on exposure to bile acids. Although bile acids are known to induce DNA damage, resistance to apoptosis through NF-κB activation, and resistance to autophagy (Fang et al., 2013), the association between bile acids and miRNA expression has never been reported except for our results (Masaoka and Suzuki, 2014). In this way, clinical epidemiological information would be important and useful to reveal novel insights of miRNA in the progression of Barrett's esophagus to adenocarcinoma.

In conclusion, on the basis of clinical importance, better risk stratification biomarkers to determine the risk of progression from Barrett's esophagus to esophageal adenocarcinoma are expected. We should deepen our knowledge of miRNA using clinical materials, hopefully with more prospective approach. The fusion of basic science and clinical science research would also be required for identifying the upstream regulation and the downstream targets of miRNAs and understanding their mode of action. These will facilitate the development of miRNA-based prevention or therapeutic strategies for esophageal adenocarcinoma.

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