Table 2
Changes in polysomnographic variables between the two time points.

	1st mean ± SD (range)	2nd mean ± SD (range)	p-value
Apnea-hypopnea index (/h)	17.1 ± 5.5 (11.2–30.2)	11.0 ± 7.2 (4.0–22.7)	0.011*
Lowest SpO2 (%)	85.4 ± 8.8 (72–98)	88.5 ± 5.8 (79–96)	0.155
Arousal index (/h)	18.8 ± 6.7 (11.1–31.5)	17.5 ± 9.3 (6.7–39.2)	0.520
Snoring index (/h)	21.9 ± 10.4 (7.2–38.3)	23.1 ± 11.5 (3.3–40.4)	0.762
Periodic leg movement index (/h)	9.2 ± 17.2 (0–45.3)	9.9 ± 14.4 (0–37.9)	0.709
Sleep efficiency (%)	77.0 ± 9.7 (59.2–94.3)	76.1 ± 12.2 (49.5–95.8)	0.731
Stage 1 (%)	17.7 ± 6.1 (6.5–25.7)	19.7 ± 7.0 (11.7–35.1)	0.502
Stage 2 (%)	57.6 ± 6.2 (46.7–65.4)	58.5 ± 8.1 (40.8–67.1)	0.773
Slow wave sleep (%)	2.0 ± 1.8 (0.0–5.7)	2.9 ± 3.0 (0–4.7)	0.376
Rapid eye movement (%)	20.1 ± 7.4 (9.4–33.3)	18.9 ± 5.6 (10.4–27.8)	0.562

* Sleep stage was calculated as a percentage (%) of total sleep time.

Table 3
Relationship between improvement in degree of polysomnographic variables and cephalometric variables.

	Apnea–hypopnea index (/h)	Lowest SpO2 (%)	Arousal index (/h)	Snoring index (/h)	Sleep efficiency (%)	Slow wave sleep (%)
Body mass index ($kg\,m^{-2}$)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Mandibular plane angle (deg.)	n.s.	n.s.	n.s.	n.s.	n.s.	R = 0.662 p = 0.037*
SNA (deg.)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SNB (deg.)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ANB (deg.)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Lower face height (%)	n.s.	n.s.	n.s.	n.s.	R = 0.742 p = 0.014*	R = 0.845 p = 0.002*
Hyoid to mandible plane (deg.)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Tongue area (mm^2)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Soft palate area (mm^2)	n.s.	n.s.	n.s.	n.s.	R = −0.809 p = 0.005*	n.s.

* $p \leq 0.05$.

sleeping better, and four patients (40%) indicated that they were waking up feeling better.

4. Discussion

There was no significant difference in the polysomnographic variables, except for AHI, obtained before and after the Silensor® therapy. These results of a low improvement in degree of polysomnographic variables in this study compared with the results of other studies [3,6,7,12] may be caused by the small number of participants which is the most obvious limitation in the study. These ten cases include three cases whose data for AHI and slow wave sleep (%) were not improved after the Silensor® therapy. However, it was difficult to detect characteristics to distinguish the responders and the non-responders to the Silensor® therapy from this study because of no significant difference in lowest SpO2, arousal index, or snoring index particularly which may make clear the treatment mechanism of Silensor® to improve AHI.

The results of a significant correlation between the improvement in degree of slow wave sleep (%) and the mandibular plane angle, as well as between the improvement in degree of slow wave sleep (%) and the lower face height also suggest that Silensor® improves the quality of sleep in OSA patients with a "long face" who have a larger mandibular plane angle than 30.7 degrees (calculated using a regression line; mean value in Japanese: 26.25 ± 6.34 degrees) or a larger lower face height than 57.1% (calculated using the regression line in Fig. 4; mean value in Japanese: 57.3 ± 0.74%). Furthermore, because of the significant correlation between the improvement in degree of sleep efficiency (%) and the lower face height, Silensor® may improve the quality of sleep in OSA patients who have a larger lower face height than 57.6% (calculated using

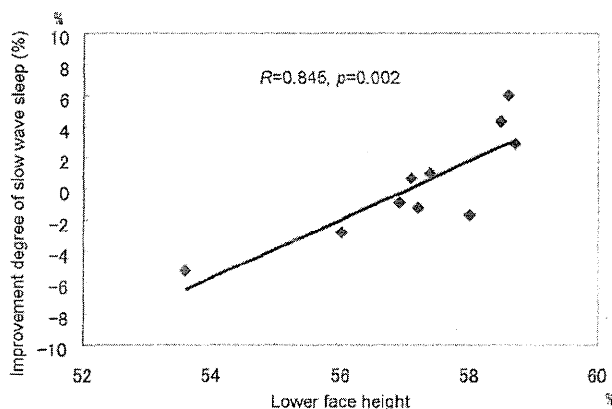

Fig. 4. Correlation between improvement in degree of slow wave sleep (%) and lower face height.

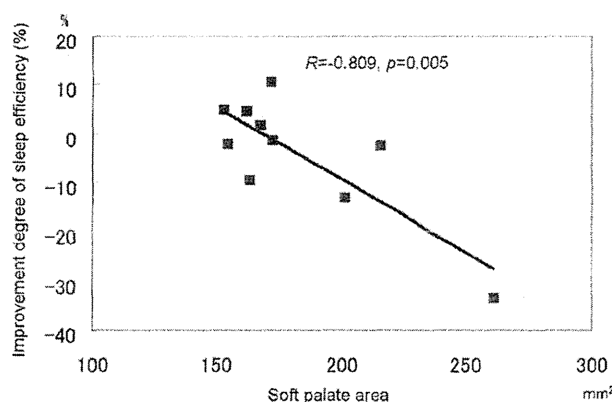

Fig. 5. Correlation between improvement in degree of sleep efficiency (%) and soft palate area.

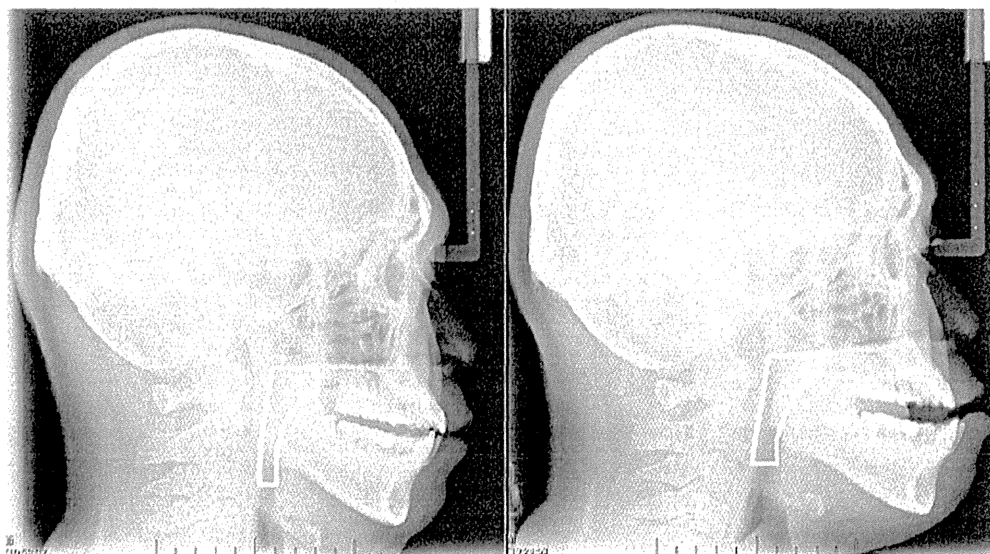

Fig. 6. The lateral cephalometric radiographs of an obstructive sleep apnea patient that were taken without Silensor® closing the mouth (left) and with Silensor® opening the mouth (right).

a regression line; mean value in Japanese: 57.3 ± 0.74%). From these findings, the Silensor® therapy may prevent the mandible of "long face" individuals from rotating downwards and back in the supine position. Özdemir et al. [13] described that an increase in gonial angle results in reduced protrusor mechanical efficiency of genioglossus muscle and relative retrodisplacement of the tongue which are associated with consequent chronic mouth breathing. Their suggestion indicates the possibility that using Silensor® for patients with larger mandibular plane angle may prevent retrodisplacement of the tongue keeping the nasopharyngeal airway space, even if the patient is a mouth open sleeper.

Previous studies [14,15] reported that OSA patients had posteriorly rotated mandibles and longer lower facial height. Battagel et al. [4] indicated that a reduced lower facial height or low mandibular plane angle was associated with a good airway response to mandibular protrusion. Based on similar results, Liu et al. [16] considered that a mandibular repositioner was likely to make the mandible of a patient with a steep mandibular angle rotate clockwise so that the genioglossus and hyoid musculature tends to be closer to the posterior pharyngeal wall. However, these studies used monobloc form MASs, accordingly the opposite findings in the favorable craniofacial features between the previous studies and our study which used the two-part semi-rigid OA are acceptable. Differently from most monobloc form MASs, the main treatment mechanism of Silensor® is thought to avoid the mandible retract during mouth opening while maintaining the mandible near the occlusal position during mouth closing. The compact form of Silensor® makes possible the maintenance of the mandible near the occlusal position during mouth closing and the advantage may be more effective for quality of sleep of patients with a "long face" by preventing mandible from rotating clockwise.

The lateral cephalometric radiographs of an OSA patient that were taken without Silensor® closing the mouth and with Silensor® opening the mouth showed slight changes in position of the mandible and in size of the nasopharyngeal airway space (Fig. 6). With Silensor® opening the mouth, the mandible moved forward 5 mm at the most and the area of the nasopharyngeal airway space showed an increase by approximately 10%. This changing range of area made us consider that Silensor® kept the nasopharyngeal airway space during sleep.

Skinner et al. [7], using a rigid but titratable MAS indicated the importance of distance from the hyoid bone to the mandible in order to predict the treatment outcome and the helpfulness of supine cephalometry. Therefore, although there was no significant correlation between the improvement in degree of polysomnographic variables and the distance from hyoid bone to mandible plane, to measure the distance from the hyoid bone to the mandible using the cephalometric films taken in the standing position in this study may be meaningless. On the other hand, their result of no cephalometric measure except for the distance from the hyoid bone to the mandible associated with an improvement in polysomnographic variables was not consistent with our result. There are no precise criteria for deciding a MAS design on facial morphology and the difference in results among studies using various types of MAS may suggest differences in the susceptible range due to the MAS design. To confirm this possibility, it is necessary to examine and compare the two types of OA using an increased sample size.

Considering the treatment mechanism of Silensor® to maintain the mandible near the occlusal position during mouth closing and to prevent mandible from rotating clockwise, Silensor® may be suitable for OSA patients with mouth breathing. Okawara et al. [17] suggested that gradual anterior titration of mandibular position reducing the nasal resistance would give OSA patients therapeutic effects, while Zeng et al. [18] reported higher levels of nasal airway resistance which is associated with mouth breathing may negatively affect treatment outcome with MAS. There is some possibility that Silensor® is more effective for OSA patients with mouth breathing, and our results showed a significant negative correlation between the improvement in degree of sleep efficiency (%) and the soft palate area. Nasal resistance is related to transpalatal resistance and larger soft palate was mentioned as a factor to induce a poor treatment response with MAS seen in OSA patients [19]. The finding concerning the area of soft palate in our study was consistent with the results of many previous studies using MASs. A larger sample size of patients would be required in order to assess the efficacy of Silensor® in managing OSA patients with mouth breathing.

Lowe et al. [20] reported the difference in craniofacial and upper airway structure between genders and Battagel et al. [21] mentioned a greater amount of mandibular protrusion in females than males. Different from most previous OSA studies, in our study, the

number of female participants was larger than that of males and that seven of the eight female participants were postmenopausal. Young et al. [22] indicated that the menopausal transition increases the risk for sleep-disordered breathing, independent of other confounding factors. These gender or age differences in our study may influence differences in findings to previous studies.

According to the participants' subjective reaction to Silensor® therapy, most participants seemed to be satisfied with the therapeutic efficacy concerning daytime sleepiness, sleeping, and the waking up feeling, and showed few side effects. From these findings, good compliance with Silensor® therapy in the short-term is expected. Previous studies [23,24] reported a high prevalence of initial side effects with MAS therapy, including temporomandibular pain (69–41%) or occlusion change (69–32%), but they also described that no side effect disturbed the continuation of treatment in the long term. On the other hand, the most common reason given for stopping use of OA was reported to be discomfort (52%) [2]. Considering the long-term continuation of OA treatment, it is still important to reduce the temporomandibular joint or occlusal discomfort arising from use of OAs. The process to make Silensor® is more convenient than the other type of MAS, therefore, Silensor® is considered to have a good performance by selecting mild or moderate OSA patients who are susceptible to the treatment on the basis of cephalometric analysis.

5. Conclusion

A significant difference was observed in AHI after 3 months of therapy using the two-part semi-rigid OA, Silensor®. Furthermore there was a significant positive correlation between the improvement in degree of slow wave sleep (%) and the mandibular plane angle, as well as between the improvement in degree of slow wave sleep (%) and the lower face height. A significant negative correlation was also observed between the improvement in degree of sleep efficiency (%) and the soft palate area. These results suggested that keeping the nasopharyngeal airway space during mouth opening improves AHI of some patients with mild or moderate OSA and has the potential of making the quality of sleep better in OSA patients with a long lower face height and a small soft palate.

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RESEARCH ARTICLE

Efficacy of Aprepitant in Patients with Advanced or Recurrent Lung Cancer Receiving Moderately Emetogenic Chemotherapy

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Abstract

Aims and Background: To evaluate the efficacy of a combination of aprepitant and conventional antiemetic therapy in patients with advanced or recurrent lung cancer receiving moderately emetogenic chemotherapy (MEC). Methods: Patients with advanced or recurrent lung cancer who were treated with MEC regimens at the Department of Respiratory Medicine, Fukuoka University Hospital, were included and classified into the following groups: control group (treatment: 5-HT3 receptor antagonists + dexamethasone) and aprepitant group (treatment: 5-HT3 receptor antagonists + dexamethasone + aprepitant). The presence or absence of chemotherapy-induced nausea and vomiting (CINV) was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.0; patients with grade 1 or above were considered positive for CINV. Food intake per day, completion of planned chemotherapy, and progression-free survival (PFS) achieved by chemotherapy were investigated. Results: The complete suppression rate of nausea in the aprepitant group was significantly higher than that in the control group ($p = 0.0043$). Throughout the study, the food intake in the aprepitant group was greater than that in the control group, with the rate being significantly higher, in particular, on day 5 ($p = 0.003$). The completion rate of planned chemotherapy was also higher in the aprepitant group ($p = 0.042$). PFS did not differ significantly, but tended to be improved in the aprepitant group. Conclusions: The aprepitant group showed significantly higher complete suppression of nausea, food intake on day 5, and completion of planned chemotherapy than the control group.

Keywords: CINV - aprepitant - complete suppression rate of nausea - food intake

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Introduction

Chemotherapy-induced nausea and vomiting (CINV) is one of the most severe adverse effects of anticancer treatments, and its prolonged manifestation can cause dehydration, electrolyte imbalance, and poor nutrition. Further, CINV reduces patients' quality of life (QOL) and can prevent the continuation of chemotherapy. Therefore, prevention of CINV and symptom management are important (Richardson et al., 1988).

Several mechanisms underlie the induction of CINV by chemotherapy. First, chemotherapeutic agents stimulate enterochromaffin cells that signal the vomiting center in the bulbar lateral reticular formation using the neurotransmitter 5-hydtroxytryptamine (5-HT) via 5-HT3 receptors in the gastrointestinal tract either directly through the vagus nerve or through the chemoreceptor trigger zone (CTZ). Second, the agent can directly stimulate the CTZ, transmitting to the vomiting center via the dopamine or 5-HT3 receptors (Navari, 2009a; Navari, 2009b). Furthermore, in a newly elucidated pathway, chemotherapeutic agents can increase secretion of substance P in the area postrema and the nuclei of the solitary tract in the medulla oblongata, which binds to neurokinin 1 (NK 1) receptor in the central nervous system. Thus, this represents a new target in antiemetic therapy (Huskey et al., 2003; Navari, 2009a; Navari, 2009b).

The risk of CINV depends on the type of chemotherapeutic agents, which are classified into 4 emetic risk groups (Kris et al., 2006). Cisplatin, the main drug for treating lung cancer, is classified as a highly emetic chemotherapy (HEC). Several clinical trials have demonstrated the efficacy of NK1-receptor antagonists in HEC (Hesketh et al., 2003; Poli-Bigelli et al., 2003; de Wit et al., 2004), and the American Society of Clinical Oncology (ASCO), Multinational Association of Supportive Care in Cancer (MASCC) and National Comprehensive Cancer Network(NCCN) guidelines recommend combined administration of 5-HT3 receptor

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antagonists, steroids, and NK1-receptor antagonists (Kris et al., 2006; Ettinger et al., 2007). In Japan, 5-HT3 receptor antagonists + steroids were previously the standard of care, because NK1-receptor antagonists had not been approved. However, the NK1-receptor antagonist aprepitant gained market approval in 2009. Since then, the Japanese antiemetic guidelines, which were updated in 2010, recommend its usage in treatment regimens including HEC (Takeuchi & Saeki, 2010).

On the other hand, there is less evidence to support the efficacy of aprepitant in treatment regimens with moderately emetogenic chemotherapy (MEC) in patients with lung and other cancers. Palonosetron, which has a long half-life (~40 h) and a high affinity and selectivity for 5-HT3 receptors, has antiemetic effects in both the acute phase and the delayed phase (after 24 h) by blocking 5-HT3 receptors (Wong et al., 1995; Rojas et al., 2008; Saito et al., 2009). Based on these results, palonosetron is recommended for use in regimens including MEC in the guidelines by American Society of Clinical Oncology (ASCO) and Multinational Association of Supportive Care in Cancer (MASCC) (Roila et al., 2010; Basch et al., 2011). Rapoport et al. investigated the effects of antiemetic therapies in 848 patients (52% with breast cancer, 20% with colorectal cancer, 13% with lung cancer, and 4.6% with ovarian cancer) who were treated with MEC and started antiemetic therapy from the first course of chemotherapy. In this a double-blind comparative study, they compared the antiemetic effects between the triple treatment (aprepitant + ondansetron + dexamethasone) and the double treatment (ondansetron + dexamethasone) groups. They found a significant improvement in antiemetic effects by adding aprepitant (Rapoport et al., 2010), suggesting its preventive effect in patients with lung cancer treated with MEC regimens.

Herein, we report the results of a retrospective study on the efficacy of aprepitant in patients with advanced and recurrent lung cancer receiving MEC.

Materials and Methods

Patient groups

Patients with advanced or recurrent lung cancer who were treated with MEC regimens at the Department of Respiratory Medicine, Fukuoka University Hospital were included and classified into the control group (receiving 5-HT3 receptor antagonists + dexamethasone) and the aprepitant group (receiving 5-HT3 receptor antagonists + dexamethasone + aprepitant). The treatment period of the first course of chemotherapy for each patient was included.

Treatment administration

5-HT3 receptor antagonists were administered by 30-min infusion prior to chemotherapy. Aprepitant was administered orally at 125 mg on day 1 prior to chemotherapy and 80 mg each on day 2 and 3. Dexamethasone was administered by 30-min infusion prior to chemotherapy in combination with the 5-HT3 receptor antagonists.

Investigation methods

The total study period was from the initiation of chemotherapy until day 5. The presence or absence of CINV was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.0. Grade 1 or higher was considered as being positive for CINV.

The amount of food intake per day was obtained as a percent. The completion rate of planned chemotherapy and the progression-free survival (PFS) achieved by the chemotherapy were also analyzed.

The statistical analysis of outcomes in both groups were performed using the χ^2 test for the complete suppression rate of nausea, 2-sided 2-sample t-tests for the amount of food intake and the completion rate of planned chemotherapy, and log-rank test for PFS. The statistical significance level was set at $p < 0.05$.

Results

The characteristics of the patients in each group are shown in Table 1. There were 27 and 25 patients in the control and aprepitant group, respectively. The mean ages were 70.7 and 65.7 years, respectively. Most of the chemotherapy regimenss were CBDCA combination therapy, and some included amrubicin. The occurrence of CINV is shown in Figure 1. Throughout the study period, the complete suppression rate of vomiting was 96% in the control group and 100% in the aprepitant group. Complete response (CR) rate was defined as the complete suppression of vomiting and no salvage therapy. CR was

Table 1. Patients Characteristics and Chemotherapy Regimens Administered to the Study Population

	Control group (n=27)	Aprepitant group (n=25)
Male	20	19
Female	7	7
Age, years (range)	70.7 (34-38)	65.7 (44-83)
Regimen		
CBDCA+PAC (+BEV)	8	3
CBDCA+GEM	6	1
CBDCA+VP-16	4	7
CBDCA+PEM (+BEV)	3	9
CBDCA+TS-1	2	3
CBDCA+DOC	0	1
Other	4	1

CBDCA, Carboplatin; PAC, paclitaxel; GEM, gemcitabine; VP-16, Etoposide; PEM, pemetrexed; TS-1, tegafur gimeracil and oteracil potassium; DOC, docetaxel, BEV, bevacizumab

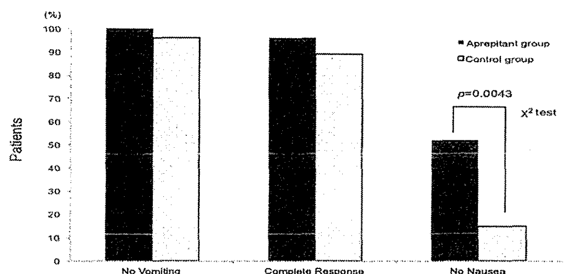

Figure 1. No Vomiting (complete suppression rate of vomiting)**, Complete Response** (defined as no emetic episodes and no use of rescue medication) **and No Nausea** (complete suppression rate of nausea) **Rates in Each of the Two Groups**

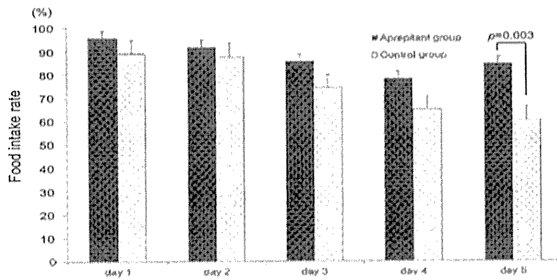

Figure 2. Food Intake Rate from Days 1 to 5 in Each of the Two Groups

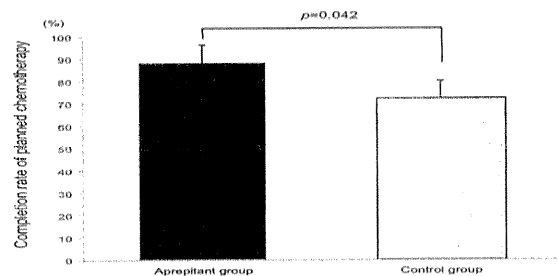

Figure 3. Completion rate of Planned Chemotherapy in Each of the Two Groups

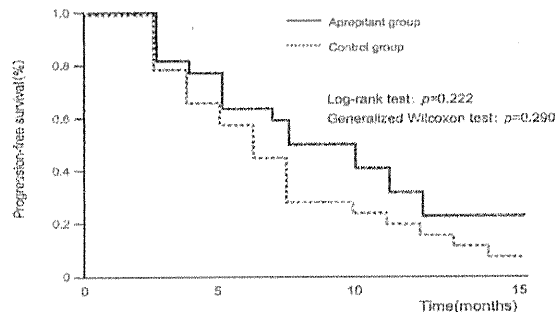

Figure 4. Kaplan-Meier PFS Curves by Treatment Arm

89% in the control group and 96% in the aprepitant group. The complete suppression rate of nausea was 14.8% and 52% in the control and aprepitant group, respectively. The aprepitant group had a significantly higher rate than the control group (p = 0.0043). The amount of food intake was greater throughout the study period in the aprepitant group, with significantly higher on day 5 in the aprepitant group (60.4% vs. 84.4%, p = 0.003) (Figure 2). The completion rate of planned chemotherapy was also higher in the aprepitant group (73.3% vs. 88.2%, p = 0.042) (Figure 3). PFS did not significantly differ, but it tended to be improved in the aprepitant group (Figure 4).

Discussion

CINV is a severe adverse effect in patients and can reduce QOL. As such, prevention and treatment of CINV are important. The present study investigated CINV during MEC treatment. MEC-induced vomiting in the acute phase is well controlled by 5-HT3 receptor antagonists (Perez et al., 1998; Jordan et al., 2007). However, delayed vomiting and nausea throughout the treatment period are still not well controlled during MEC, causing negative attitudes towards treatment and hindering the continuation of chemotherapy. Although steroids are recommended for treating delayed nausea and vomiting, their side effects remain a concern for many clinical oncologists (Vardy et al., 2006). On the other hand, anticipatory nausea and vomiting can occur by 'conditioning' mechanisms in patients who have experienced nausea and vomiting from chemotherapy (Morrow & Morrell, 1982). Anticipatory vomiting occurs in 11% of patients, and anticipatory nausea occurs in 29% of patients who receive chemotherapy (Andrykowski, 1988). In general, antiemetic agents cannot treat anticipatory nausea and vomiting, and the best countermeasure is to avoid nausea and vomiting from the beginning of chemotherapy (Andrykowski, 1988; Morrow et al., 1991). This retrospective study evaluated the efficacy of aprepitant in combination with conventional antiemetic therapy in patients receiving MEC. We found no significant difference in the complete suppression rate of vomiting or the CR rate between the control group and the aprepitant group. However, the complete suppression rate of nausea was significantly higher in the aprepitant group. These results suggest that nausea is not completely suppressed with conventional 5-HT3 receptor antagonists + dexamethasone in patients receiving MEC, and that adding aprepitant effectively suppresses nausea. However, it should be noted that the suppression rate remained at 52%; 85% of which incorporated palonosetron as the 5-HT3 receptor antagonist, suggesting that triplet aprepitant + palonosetron + dexamethasone is effective in completely suppressing nausea associated with MEC.

Physical fitness is important for administering chemotherapy as scheduled. The amount of food intake during the treatment period is especially important for the continuation of therapy. In this study, we compared the amount of food intake during the first 5 days from the beginning of the chemotherapy between the groups. The amount of food intake was greater in the aprepitant group throughout the 5-day period with a significant difference on day 5. Patients often demonstrate a decline in the amount of food intake on days 4 to 5, as was the case in this study. Although the amount of food intake declined during this period, the difference between the control group and the aprepitant group grew larger. Indeed, there was even a tendency towards recovery in the amount of food intake in the aprepitant group on day 5. Furthermore, the aprepitant group showed a significantly higher completion rate of planned chemotherapy compared with the control group. We hypothesize that the treatment could be continued, because the increased food intake sustained a higher level of physical fitness. In addition, we assessed the antitumor effect of chemotherapy by progression free survival (PFS). Although there was no significant difference in PFS between the groups, this could have been due to the small number of patients. There was a tendency toward a longer PFS in the aprepitant group, suggesting a contribution to the increased treatment completion rate. This result also was considered to have contributed significantly as a result of the treatment plan can be carried out by maintaining of food intake.

Aprepitant used in combination with standard antiemetic therapy (5-HT3 receptor antagonist and corticosteroid) was well tolerated and effective in preventing CINV associated with Moderate moderate

emetogenic antitumor agents of in Japanese lung cancer patients.

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RESEARCH ARTICLE

A New Cancer Cell Detection Method Using an Infectivity-enhanced Adenoviral Vector

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Abstract

Cytological examination is widely used as a diagnostic tool because of the ease of collecting cells from the involved area. However, the diagnostic yield of cytological examination is unsatisfactory; the reasons include sampling error, poorly prepared samples, small numbers of malignant cells, and low grades of cellular atypia. In this study, we focused on the high infectivity of adenovirus towards epithelial cells and applied the luciferase-expressing adenoviral vector to a new cancer cell detection tool. In addition, adenoviral infectivity was enhanced by modifying viral fiber proteins. The sensitivity of the diagnostic tool was tested using the NCI-H1299 lung cancer cell line, and validated in body fluid samples from cancer patients with a variety of etiology. Results showed that the adenovirus efficiently transfected NCI-H1299 with high sensitivity. Only 10 cancer cells were sufficient for detection of luciferase signals. In body fluid samples, the adenovirus confirmed the diagnosis for malignant and benign cancer, but not in non-epithelial cell derived samples. This study provides proof-of-concept for a more reliable and sensitive diagnostic tool for epithelium-derived cancer.

Keywords: Adenoviral vector - cytology - luciferase assay - malignant effusion

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Introduction

The diagnosis of malignant disease is made by cytological or histological examination of clinical samples obtained from patients. Histological examination requires a certain amount of tissue, and using an invasive procedure to obtain this type of sample is inevitable. In contrast, cytological examination only requires small clinical samples collected by non-invasive techniques, including body fluids, lavage of targeted area, and needle aspiration of lymph nodes. This approach is commonly used in the case of poorly conditioned patients unable to tolerate invasive procedures. Clinical samples suitable for cytological examination include urine, sputa, pleural effusion, ascites, fine needle aspirations of lymph nodes or lesions, and lavage fluid from involved areas, all of which can be obtained by minimally invasive procedures. The malignant pleural effusion (MPE) defines effusion from direct infiltration of the pleura by cancer cells. The annual incidence of MPE in the United States is about 150,000. More than 75% of MPEs are caused by neoplasms of the lung, breast, or ovary or by lymphoma (Hausheer and Yarbro, 1987; Henschke et al., 1991; Martinez-Moragon et al., 1998; Antunes and Neville, 2000; Heffner and Klein, 2008). However, the sensitivity of cytological examination in the detection of cancer cells is only approximately 65% (Nance et al., 1991; Starr and Sherman, 1991; Woenckhaus et al., 2005; Benlloch et al., 2006; Sriram et al., 2011). Additional studies could complement standard cytology. Combinations of tumor markers, however, could help select patients with negative pleural effusion cytologic results for additional diagnostic studies. Moreover, additional immunostaining or other specific staining improves the diagnostic yield to some extent, though the promptness of diagnosis is adversely impaired (Kuenen-Boumeester et al., 1996 ; Porcel et al., 2004; Lee and Chang, 2005; Shitrit et al., 2005; Westfall et al., 2010 ; Su et al., 2011). Methods such as fluorescence in situ hybridization analysis, image analysis cytometry, and PCR, are more sensitive than standard cytologic studies (Fieglure, 2005; Holloway et al., 2006; Sriram et al., 2011). Investigations are underway to determine if the detection of aneuploidy adds diagnostic value and meaningful therapeutic consequences to standard effusion analysis for the detection of MPE (Fieglure, 2005; Osterheld et al., 2005; Northup et al., 2007; Sriram et al., 2011). In this study, we focused on effective gene transduction into cancer cells by an adenoviral vector and conceived of

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the application of the viral vector as a diagnostic tool in malignant diseases. Serotype 5 adenoviral vector is used in basic research as an effective tool for gene transduction into human cells. In clinical trials, the adenoviral vector containing various genes has been used for gene therapy of malignant disease. Because serotype 5 adenovirus infects human cells via the coxsackie-adenovirus receptor (CAR) and integrin on the cell membrane, infectivity depends mainly on the expression status of these molecules. The efficiency of Ad5 (serotype 5 adenovirus) gene transfer may closely correlate with the cell surface density of its primary receptor, coxsackie and adenovirus receptor (CAR) (Zabner et al., 1997; Kaner et al., 1999; Nalbantoglu et al., 2001). Unfortunately, the expression of CAR is highly variable, and is often low on lung and other primary cancer cells, which results in relative resistance to Ad5 infection (Hemmi et al., 1998; Miller et al., 1998; Takayama et al., 2003). To overcome this limitation, we have developed a new chimeric Ad5 vector, Ad5/3, which contains a chimeric fiber protein possessing a serotype 3 knob. In addition, our previous study has revealed that a distinct Ad3 (serotype 3 adenovirus) receptor exists in various cancer cells based on a novel knob binding assay, and that the Ad5/3 chimeric vector is retargeted to the Ad3 receptor with higher gene transfer efficiency than Ad5 (Kanerva et al., 2002 ;Kawakami et al., 2003). We also confirmed that this Ad5/3 showed higher infectivity toward various cancer cells, especially ovarian and lung cancer cells (Kanerva et al., 2002 ;Kawakami et al., 2003). The present study proposes a new approach to cancer detection based on gene transfer. Combining this new diagnostic tool with conventional cytology can considerably improve the diagnostic yield.

Materials and Methods

Cell culture

The NCI-H1299 lung cancer cell line and HEK293 adenoviral-transformed human embryonic kidney cell line were obtained from ATCC (American Type Culture Collection, Manassas, VA). Cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 5% fetal bovine serum and incubated at 37 °C in 5% CO2.

Artificial malignant fluid

NCI-H1299 cells were used to prepare the artificial malignant fluid sample because they were confirmed to be susceptible to adenoviral infection in our previous experiment because preliminary experiments showed that these cells are susceptible to adenoviral infection. The artificial malignant fluid was prepared by diluting human peripheral blood (collected from healthy donors) with culture medium (5% v/v) (A.P.Fishman et al., 2008), and a range of NCI-H1229 cells were added to it (10-1000 cells) to determine the cutoff point of diagnostic value.

Adenoviral vectors

Recombinant adenoviral vectors expressing firefly luciferase were constructed through homologous recombination in Escherichia coli using the AdEasy system (He et al., 1998). A cytomegalovirus immediate early promoter derived from plasmid pCEP4 (Invitrogen, Carlsbad, CA) was placed next to the firefly luciferase gene in an Ad E1 shuttle vector, recombined with the E1- and E3-deleted adenoviral backbone vector pAdEasy 1, and transfected into HEK293 cells by standard techniques to form Ad5CMVLuc (Figure 1) (He et al., 1998). The luciferase gene was derived from pGL3 Basic (Promega, Madison, WI). A modified adenoviral vector was also used for enhancement of infectivity to cancer cells in this experiment. In this modified adenovirus, only the knob domain of the conventional serotype 5 adenovirus was replaced with that of serotype 3 adenovirus as reported previously (Kawakami et al., 2003). The luciferase-expressing adenovirus based on this chimeric adenovirus (Ad5/3CMVLuc) was generated in the same fashion described above. The adenoviruses were propagated in the adenovirus-packaging cell line HEK293 and purified by double CsCl density gradient centrifugation, followed by dialysis against phosphate-buffered saline with 10% glycerol.

Luciferase assay

NCI-H1299 cells were plated in 12-well plates in triplicate at a density of 1 × 105 cells/well. After overnight culture, the cells were infected with Ad5CMVLuc or Ad5/3CMVLuc at 1 viral particle (vp)/cell or 10 vp/cell in DMEM with 2% FCS for 3 h and maintained in complete medium (Adachi et al., 2001). The infected cells were harvested and treated with 100 μL of lysis buffer (Promega, cat #E153A) after a 2-day culture. A luciferase assay (Luciferase Assay System, Promega) and a luminometer (GENE LIGHT 55A, Microtec Nition, Tokyo, Japan) were used for the evaluation of luciferase activity in the infected cells. Luciferase activity was normalized by protein concentration in the cell lysate (Bio-Rad DC Protein Assay Kit, Hercules, CA).

In the fluid samples, a fixed dose of 1 × 104 pfu of Ad5/3CMVLuc was mixed into the fluid with or without a red blood cell (RBC) lysis procedure, because RBCs can prevent infection by adenovirus (Lyons et al., 2006). The infected samples were cultured in the flask for 48 h. Collected cells were then applied to the luciferase assay in the same fashion described above. Luciferase activity was expressed as an arbitrary unit. To remove RBCs in the fluid, 10 mL of lysis buffer (RBC Lysis Buffer, eBioscience, San Diego, CA) was mixed in 10 mL of fluid sample and incubated for 10 min at room temperature. The lysis reaction was stopped by adding 30 mL of PBS. After the RBC lysis procedure, the cells in the fluid sample were spun down at 4 °C and applied to the luciferase assay.

Body fluid samples

Body fluid samples consisting of 15 pleural effusions and 10 ascites samples were obtained from 25 patients at Kyushu University Hospital. All patients provided consent (one sample was used as a negative control) for cytological examination of their samples at the central clinical laboratory. Cytology results were classified

into positive, negative, or inconclusive. In addition to cytological evaluation, the pleural effusion and ascites samples were clinically evaluated for malignancy. For example, some cytologically negative pleural or peritoneal effusions were found to be malignant effusions based on the following findings: detection of malignant cells after repeated cytological examination, increasing amounts of effusion in the clinical course with intrapleural or intraperitoneal disseminated lesions confirmed by imaging studies, or decreasing amounts of effusion following anticancer chemotherapy.

Statistical methods

Data represent mean values from three separate readings with the error bars showing standard deviation. Data shown was consistent for two or more repeat studies performed on different days. Continuous measures were compared between groups using two-sample unpaired t tests.

Results

Infectivity of Ad5 and Ad5/3 in NCI-H1299 cells

NCI-H1299 cells derived from human lung cancer were infected by Ad5CMVLuc or Ad5/3CMVLuc at various m.o.i. Approximately 48 h after infection, cells were collected and applied to the luciferase assay as described in Materials and Methods. As shown in Figure 2, the cells infected by Ad5/3CMVLuc exhibited higher luciferase activity compared with those infected by Ad5CMVLuc. We have observed that ovarian and lung cancer cells were susceptible to Ad5/3 infection which is in agreement with published literature (Kanerva et al., 2002). Based on these results, NCI-H1299 cells were used in an artificial effusion in the next step.

Luciferase activity in artificial effusions containing cancer cells

Luciferase activities in artificial malignant effusions containing various numbers of cancer cells are depicted graphically in Figure 3. First, artificial effusions were applied to luciferase assays without RBC lysis procedures. The luciferase activity in the sample mixed with 10, 30, and 100 cancer cells showed a similar value and no significant difference compared with that the negative control (sample not containing any cancer cells). The sample containing 300 or more cancer cells expressed significantly higher luciferase activity. The cutoff point was between 100 and 300 cancer cells in the artificial effusion. Because RBCs have a negative charge on the cell surface, they may nonspecifically interfere with viral infection of cancer cells. In the next step, the RBC lysis procedure was performed prior to adenoviral infection. After the removal of RBCs, adenovirus efficiently infected cancer cells and expressed a 1- to 2-log unit higher luciferase activity in each sample, as shown in Figure 3. The luciferase activity also correlated with the number of cancer cells mixed in the artificial effusion. The high sensitivity of this diagnostic technique is evidenced by the significant luciferase signal detected with only 10 cancer cells in RBC-free samples. The sample containing only 10 cancer cells showed significant luciferase activity when compared with the control.

Luciferase activity in clinical samples

This new cancer cell detection tool, which uses artificial effusions, showed a promising result that urged us to check its utility in the clinical setting. A similar experiment was performed with body fluid samples obtained from cancer patients. Table 1 shows the clinical background of each sample, including the primary malignant disease, pleural effusion or ascites, results

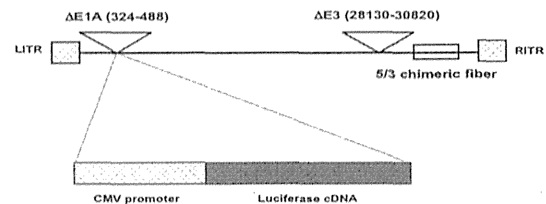

Figure 1. Schematic Diagram of 5/3 Chimeric Adenovirus Construction. This vector was constructed from an E3 region-deleted Ad5 backbone, does not contain the Ad E1A promoter region (324 bp–488 bp of the Ad genome), and has modified fiber genes that contain an Ad5 shaft region and Ad3 knob region (647 bp–1208 bp of accession no. X01998 M12411).

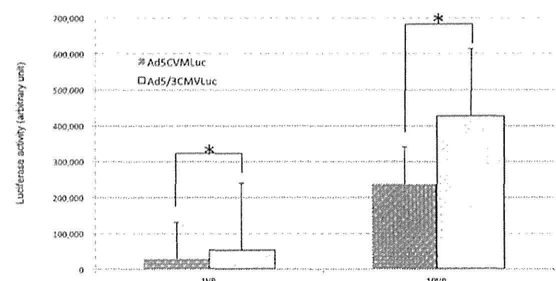

Figure 2. Luciferase Assay after Infection by Ad5 and Ad5/3 Viruses. NCI-H1299 cells were infected with equal amounts (1 vp/cell or 10 vp/cell) of Ad5Luc or Ad5/3Luc. Approximately 48 h later, luciferase activity was measured. The graph represents the average of triplicate samples. The average background luciferase activity was subtracted from all experimental values. Difference was assessed using unpaired t test; *P < 0.05

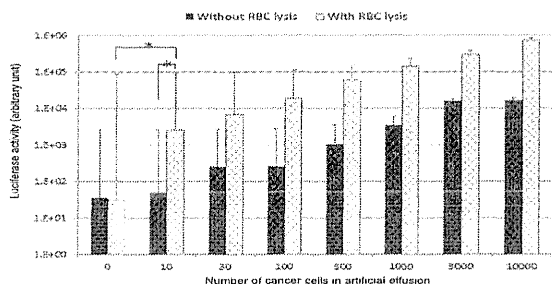

Figure 3. Luciferase Activities in Artificial Malignant Effusions Containing Various Numbers of Cancer Cells. After the removal of RBCs, adenovirus efficiently infected cancer cells and expressed 1- to 2-log unit higher luciferase activity in each sample. Difference was assessed using unpaired t test: **P <0.05, *P < 0.001

Table 1. Luciferase Activities in Body Fluid Samples and their Clinical ackground

Sample#	Primary disease	Effusion	Cytological diagnosis	Clinical diagnosis	Luciferase activity
1	Ovarian cancer	Ascites	Positive	Malignant	9691850
2	Ovarian cyst	Ascites	Negative	Benign	8392006
3	Esophageal cancer	Ascites	Positive	Malignant	4973694
4	Lung cancer	Pleural effusion	Positive	Malignant	3367039
5	Hepatic caner	Ascites	Negative	Malignant	1781689
6	Colon cancer	Ascites	Negative	Malignant	1502799
7	Ovarian cancer	Pleural effusion	Negative	Malignant	345152
8	Lung cancer	Pleural effusion	Negative	Malignant	323605
9	Breast cancer	Pleural effusion	Positive	Malignant	100029
10	Dermoid cyst	Ascites	Negative	Benign	30406
11	Lung cancer	Pleural effusion	Positive	Malignant	3636
12	CML	Pleural effusion	Positive	Malignant	1330
13	Ovarian cyst	Ascites	Negative	Benign	666
14	Pnemonia	Pleural effusion	Negative	Benign	524
15	Colon cancer	Ascites	Negative	Benign	488
16	Pulmonary tuberculosis	Pleural effusion	Negative	Benign	235
17	Heart Failure	Pleural effusion	Negative	Benign	166
18	Lung cancer	Pleural effusion	Suspicious	Benign	65
19	Heart Failure	Pleural effusion	Negative	Benign	63
20	Liver chirosis	Ascites	Negative	Benign	56
21	Renal Failure	Pleural effusion	Negative	Benign	52
22	Burkitt lymphoma	Pleural effusion	Positive	Malignant	45
23	Malignant mesothelioma	Pleural effusion	Negative	Malignant	37
24	Rectal cancer	Ascites	Negative	Benign	30
25	Pneumonia	Pleural effusion	Negative	Benign	23

of cytological examination, malignant or benign status based on clinical decision, and luciferase activity. Twelve samples were found to be clinically malignant, while 5 were negative. Of the 12 clinically malignant samples, 7 were cytologically positive, in which showed a sensitivity of 58% with the conventional method. However, 9 samples were found to be positive by using the new method, when 2,474 arbitrary units in the luciferase activity was set as the cutoff point based on the result obtained with artificial effusions. Out of the 13 clinically negative samples, 12 samples were cytologically negative and 1 was unclear (pseudo-positive). By using the new method, 11 samples were found to be negative while 2 were positive. The sensitivity and specificity of the new method was 75% and 79% ($p=0.006$: chi-square test) respectively, which was better than that of the conventional cytological examination. Therefore, this new method showed better sensitivity and exhibits some improvement in specificity compared to conventional cytological examination.

Discussion

The diagnosis of a malignant disease is confirmed by pathological examination of clinical samples obtained from patients. Cytological examination is widely used because of the ease of collecting cells from the involved area. However, the diagnostic yield of cytological examination is unsatisfactory. Diagnostic accuracy of effusion cytology depends on the volume of liquid examined, the type of preparation and staining, the experience of the examiner, and the number of sufficient specimens investigated. However, the cytological interpretation of fluids can be challenging, and its diagnostic accuracy is limited (Garcia-Bonafe and Moragas, 1996). To supplement the morphological examination of doubtful cytological effusions, immunocytochemistry and a variety of molecular methods have frequently been applied (Motherby et al., 2002; Davidson, 2004; Sriram et al., 2011). However, the sensitivity and specificity of these techniques were not superior to current immunocytochemistry (Motherby et al., 1999; Sriram et al., 2011).

As expected, Ad5/3 chimeric vector showed good performance in cancer cell detection and better sensitivity compared to conventional cytological examination. This technique is highly reliable for body fluids and requires prior RBC lysis for blood samples. One of the limitations of this technique is decreased infectivity due to RBC interference, probably due to some interactions with RBC in the blood samples (Lyons et al., 2006). To avoid RBC interference, RBCs contained in samples must be lysed prior to viral infection. This additional procedure entails extra work for the cytologist. Another feature of this method is low infectivity towards malignant cells derived from non-epithelial tissue. As shown in Table 1, there were 3 pseudo-negative samples (No. 12,22, and 23), showing very low luciferase activities despite the presence of many malignant cells in the samples. Interestingly, the primary diseases of these 3 pseudo-negative samples were leukemia, lymphoma, and mesothelioma, respectively. This finding may suggest a low infectivity of adenovirus in non-epithelial cells. Therefore, this method is suitable only for the detection of cancer cells derived from epithelial tissues.

Two samples that were found to be cytologically negative showed a high luciferase activity (pseudo-positive). This could be because of a dermoid cyst (Sample 10), which is a cystic teratoma that contains developmentally mature skin, complete with hair follicles

and sweat glands, sometimes clumps of long hair, and often pockets of sebum, blood, fat, bone, nails, teeth, eyes, cartilage, and thyroid tissue. We speculate that some epithelial cells exuded from the cyst into the ascites, and were infected by the adenovirus. In the case of sample 2, it is not clear that the ovarian cyst produced ascites; however, it could be similar to sample 10. Such pseudo-positive reaction to cystic tumor needs to be explored in our future studies. In the case of sample 11, the luciferase activity is low because the number of cancer cells in effusion is very low at the cytologic examination. In the current study, if a clinical condition suggested malignancy, we recommended a cytology re-examination. In addition, the low sample numbers used in this study may limit the validity of the results; a higher number of samples will be used for future studies.

In this method, the luciferase gene was driven by the CMV promoter, enabling nonspecific luciferase expression in normal as well as in malignant cells. Because floating cells in ascites and pleural effusions are mainly from blood or mesothelial tissues, there may be few epithelial cells in addition to cancer cells. Therefore, ascites and pleural effusion are suitable for this method. However, lavage fluid, such as bronchial washing solution, contains many normal epithelial cells. These normal cells deteriorate the signal-noise ratio by nonspecific gene expression. To improve expression in cancer cells specifically, tumor-specific promoters are preferred to the CMV promoter. As a part of our future studies, we will examine the specificity with the tumor-specific promoter.

In this study, we focused on efficient adenovirus-mediated gene transfer into epithelial cells and demonstrated the usefulness of the adenoviral vector as a cancer cell detection tool in malignant effusions. Although there is room for improvement of this method in the clinical setting, they are surmountable hurdles. In any case, the technique used in this study is definitely useful to detect malignancy in cytologically negative effusions. Tumor cell detection in effusions can be significantly improved by combining this technique with standard cytology. This finding should help to improve tumor diagnosis and staging.

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