the blood in various diseases including sepsis, trauma, stroke, autoimmune disorders, and several cancers [15–20]. This cf-DNA is thought to be derived from necrotic and/or apoptotic cells [21]. Recent articles have suggested that NETs and cf-DNA are related [15,16]. In these reports, cf-DNA was quantified directly in plasma, and the cf-DNA in plasma was treated the same as NETs in blood. However, it remains unknown whether cf-DNA is derived from NETs.

Citrullination of histone H3 is considered to be involved in NET formation in vitro. Neutrophils show highly decondensed nuclear chromatin structures during NETosis, and hypercitrullination of histone H3 by peptidylarginine deiminase 4 (PAD4) plays an important role in chromatin decondensation [14,22,23]. Inhibition of PAD4 prevents citrullination of H3 and NET formation [23]. Thus, measuring the presence of citrullinated histone H3 (Cit-H3) in conjunction with the presence of NETs may help clarify the kinetics of the response of NETs to systemic stress.

In preliminary studies, we recently identified NETs immuno-cytochemically in sputum and blood smear samples from intensive care unit (ICU) patients [24,25], whereas NETs could not be detected in blood smears from healthy volunteers [25].

In the present study, we used immunofluorescence to prospectively explore the existence of NETs and Cit-H3 in the blood of critically ill patients hospitalized in an ICU.

The respiratory tract is considered one of the most vulnerable places for bacterial invasion of the body, and NETs might start to be produced in response to pathogens before infection is completely apparent. Therefore, in this study we evaluated the presence of bacteria by Gram staining in tracheal aspirate as the preclinical stage of manifested infection to highlight its relationship with the induction of NETs in blood. The purpose of this study was to evaluate the relationships between NET or Cit-H3 and various clinical and biological parameters.

Materials and Methods

Patients and Setting

This study was a prospective observational study and was approved by the Ethics Committee of Osaka University Graduate School of Medicine. The institutional review board waived the need for informed consent. From April to June 2011, we examined blood samples collected from all patients who required intubation at the time of admission into the ICU of the Trauma and Acute Critical Care Center at the Osaka University Hospital (Osaka, Japan).

Evaluation of Clinical Background and Severity of Illness

Age, sex, Acute Physiological And Chronic Health Evaluation (APACHE) II score, and Sequential Organ Failure Assessment (SOFA) score were recorded at the time of admission. Systemic inflammatory response syndrome (SIRS) was diagnosed at the time of admission on the basis of the criteria for SIRS defined by the American College of Chest Physicians/Society of Critical Care Medicine Consensus [26]. At admission, the blood samples were analyzed to obtain the following laboratory data: white blood cell (WBC) count and concentrations of lactate, IL-8, TNF-α, HMGB1, and cf-DNA. WBC count was measured by an automated hematology analyzer (KX-21N; Sysmex, Hyogo, Japan). Lactate concentration was measured by a blood gas analyzer (ABL 835 Flex; Radiometer, Brønshøj, Denmark). The serum levels of IL-8 (R&D Systems, Minneapolis, MN, USA), TNF-α (R&D Systems), and HMGB1 (Shino-Test Corporation, Tokyo, Japan) were measured by enzyme-linked immunosorbent assay (ELISA) kits, and cf-DNA concentration was quantified

using the Quant-iT PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Immunofluorescence Analysis to Identify the Presence of NETs and Cit-H3

For histological analysis, each blood sample collected at the time of admission to the ICU was immediately smeared in a thin layer on a glass slide. After drying, the specimens were stored at −80°C until immunostaining was performed. We confirmed that this sample preparation method did not induce additional generation of NETs or citrullination of histone H3 using neutrophils isolated from healthy donors on the smear (Fig. S1). To identify NETs, DNA and histone H3, the main components in NETs, were visualized simultaneously by immunofluorescence, and Cit-H3 was also detected using a specific antibody as follows. The sample on the glass slide was fixed with 4% paraformaldehyde for 30 min, washed with phosphate-buffered saline (PBS) (pH 7.4), and then blocked with a solution containing 20% Block-Ace (Dainippon-Sumitomo Seiyaku, Osaka, Japan) and 0.005% saponin in PBS for 10 min. The samples were then incubated for 60 min with the primary antibody as follows: anti-human histone H3 mouse monoclonal antibody (diluted 1:100) (MABI0001; MAB Institute, Inc., Hokkaido, Japan) and anti-human Cit-H3 rabbit polyclonal antibody (1:100) (ab5103; Abcam, Cambridge, UK). After washing in PBS, each primary antibody was visualized using secondary antibodies coupled to 1:500 Alexa Fluor 546 goat anti-mouse IgG (Invitrogen) and 1:500 Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). The primary and secondary antibodies were diluted with 5% Block-Ace and 0.005% saponin in PBS. After incubation for 60 min with the secondary antibodies, the specimens were washed with PBS, and the DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) in PBS for 5 min. All procedures were performed at room temperature. The specimens were analyzed using a confocal laser-scanning microscope (BZ-9000; Keyence Corporation; Osaka, Japan).

The validity of immunostaining was ensured by the negative results of control experiments in which whole mouse or rabbit IgG (Abcam) was used instead of primary antibodies or primary antibodies were omitted in the procedure (Fig. S2). In addition, neutrophils stimulated with phorbol myristate acetate from healthy donors were used as a positive control for immunostaining (Fig. S3).

In the preliminary experiments, string-like structure extending from the cell body, which was positive for DNA and histone, was exclusively also positive for neutrophil elastase (Fig. S4). Hence, we considered the extracellular component that is double-positive for DNA and H3 to be a NET. The production of NETs and the specific expression of the citrullination of histone H3 in neutrophils were confirmed using anti-CD66b antibody (Fig. 1). Diff-Quik staining revealed the presence of a variety of blood cells in the smears (Fig. S5).

For the purpose of estimating the presence of NETs and the occurrence of citrullination of histone H3 concurrently, triple staining for DNA, H3, and citrullinated H3 was performed in this study. Samples were considered negative for the presence of NETs or Cit-H3 if cells harboring NETs or Cit-H3 were not identified in 300 neutrophils by immunostaining. If at least one of NETs and Cit-H3 was positive in the smear according to the definition mentioned above, the corresponding patient was classified into the "NET- and/or Cit-H3-positive" group.

Detection of the presence of bacteria in tracheal aspirate

Aspiration is defined as the inhalation of oropharyngeal or gastric contents into the larynx and lower respiratory tract, and
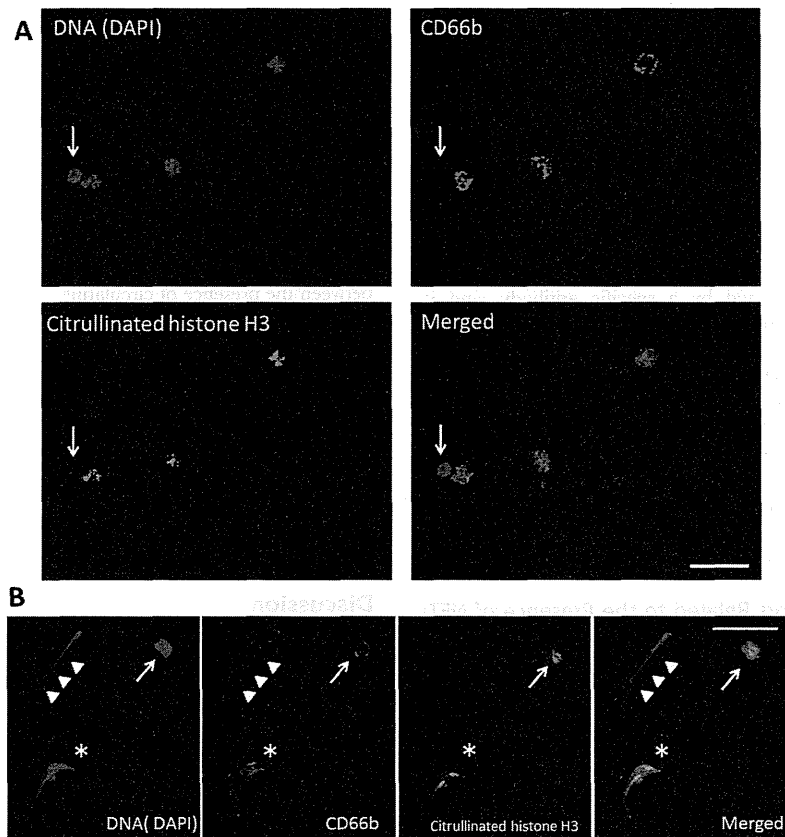
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Figure 1. Representative images of immunostaining using anti-CD66b antibody in the blood smear sample from a critically ill patient. Triple staining by DAPI, anti-CD66b antibody, and anti-citrullinated histone H3 was performed using the blood smear sample obtained from a critically ill patient. A. The CD66b-positive cells were subjected to citrullination of histone H3 in their nuclei. Citrullination of histone H3 was not detected in the CD66b-negative cell (arrow). B. Arrow indicates the occurrence of citrullination of histone H3 in a neutrophil that had immunoreactivity against CD66b. Arrowheads indicate NETs stained with CD66b, whose appearance was of a string-like structure extending from the cell body. Asterisk indicates a neutrophil that was beginning to release NETs from its ruptured cell body. Interestingly, freshly produced NETs (asterisk) held immunoreactivity against citrullination of histone H3. In contrast, elongated NETs (arrowheads) were not stained with anti- citrullinated histone H3 antibody. Blue, DAPI; Red, CD66b; Green, citrullinated histone H3. (Magnification ×400). Scale bar; 50 μm.
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aspiration pneumonia is an infectious process caused by the inhalation of oropharyngeal secretions that are colonized by pathogenic bacteria [27]. The presence of bacteria in tracheal aspirate by Gram staining is regarded as part of aspiration that favors the development of infection. In this study, we evaluated the presence of bacteria in tracheal aspirate as the preclinical stage of manifested infection. To screen for the presence of bacteria in tracheal aspirate, an aspirated sputum smear was also prepared independently from immunostaining at the time of each patient's admission to the ICU. For Gram staining, the smear was dried, stained with crystal violet (Merck KGaA, Darmstadt, Germany) followed by iodine (Merck KGaA), washed with 99.5% ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and stained with Safranin (Merck KGaA). Images were captured on an optical microscope system (ECLIPSE 50i; Nikon Instruments Inc., Tokyo, Japan).

Statistical Analysis

Continuous variables are presented as the median and interquartile range (IQR). The Wilcoxon rank-sum test and Pearson's chi-square test were used to compare two patient groups.

Single and multiple logistic regression analyses were used to identify associations between the presence of NETs and/or Cit-H3 and the clinical and biological parameters studied. A p-value of < .05 was considered significant. All statistical analyses were performed using JMP 9.0.2 (SAS Institute Inc., Cary, NC, USA) and reviewed by a statistician.

Results

Patient Characteristics

During the study period, 263 patients were admitted to the ICU; 49 of these 263 patients were intubated patients and were included in this study. We excluded patients with cardiopulmonary arrest (CPA) who could not be resuscitated on admission. The patients' characteristics are shown in Table 1. The study group comprised 29 men and 20 women with a median age of 66.0 (IQR, 52.5–76.0) years. The median APACHE II score was 18.0 (IQR, 12.5–21.5), and the median SOFA score was 5.0 (IQR, 4.0–8.0). Thirty-eight patients (77.6%) were diagnosed as having SIRS, and 22 patients (44.9%) were judged as positive for "the presence of bacteria in tracheal aspirate". Thirty-six patients (73.5%)

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survived and 13 patients died. The ICU mortality rate of intubated patients during this study period was 26.5%. The median WBC count was 10,900/µL (IQR, 8215–14,915/µL). The diagnoses included trauma (n = 7, 14.3%), infection (n = 14, 28.6%), resuscitation from CPA (n = 8, 16.3%), acute poisoning (n = 4, 8.1%), heart disease (n = 4, 8.1%), brain stroke (n = 8, 16.3%), heat stroke (n = 2, 4.1%), and others (n = 2, 4.1%) (Table 2).

Presence of NETs and Cit-H3 in the Bloodstream

NETs were identified as extracellular string-like structures that were simultaneously immunoreactive for DNA and histone H3 (Fig. 2). Cit-H3 was detected by a specific antibody, and its presence was confirmed to be located inside lobulated nuclei and histone H3 (Fig. 3). In the blood smears surveyed in this study, we identified NETs in 5 patients and Cit-H3 in 11 patients (Table 2). Both NETs and Cit-H3 were identified concurrently in one patient with infection. We detected the presence of circulating NETs and/or Cit-H3-positive cells in samples from patients with infection (4/14, 28.6%), resuscitation from CPA (5/8, 62.5%), acute poisoning (1/4, 25.0%), brain stroke (3/8, 37.5%), and heat stroke (1/2, 50.0%). We found no NETs or Cit-H3-positive cells in samples from patients with trauma (0/7) or heart disease (0/4).

Identification of Factors Related to the Presence of NETs and Cit-H3 in the Bloodstream

We tried to identify the factors that are related to the presence of NETs or Cit-H3 in the bloodstream. We first examined clinical parameters recorded at the time of admission including age, APACHE II and SOFA scores, number of patients who presented with SIRS or with the presence of bacteria in tracheal aspirate, and biological parameters such as the total WBC count and concentrations of lactate, IL-8, TNF-α, HMGB1, and cf-DNA. We also recorded the number of survivors. We compared these variables between the patients positive or negative for NETs and/or Cit-H3. The results are shown in Table 3. Among the factors evaluated in this research, only "the presence of bacteria in tracheal aspirate" differed significantly between the NET- and/or Cit-H3-positive and -negative groups ($p<.01$, Wilcoxon rank-sum test and Pearson's chi-square test). The other factors were not significantly related to the presence of NETs and/or Cit-H3. In patients classified into two groups based on the presence or absence of bacteria in tracheal aspirate, the occurrence rate of NETs and/or Cit-H3 was significantly higher in "the presence of bacteria in tracheal aspirate" (BTA (+)) group (11/22, 50.0%) than

in "the absence of bacteria in tracheal aspirate" (BTA (−)) group (4/27, 14.8%) ($p<.01$) (Table S1). In patients with SIRS on admission, there was a trend toward greater expression of NETs and/or Cit-H3 ($p = .079$) (Table S2).

Logistic regression analysis was performed to identify the factors related to the presence of NETs and Cit-H3 in the bloodstream. The results of single logistic regression analysis of factors associated with the presence of NETs and Cit-H3 are shown in Table 4. Only BTA (+) at the time of intubation was a significant factor associated with the presence of NETs and Cit-H3 ($p = .0112$). Although there were indications of a trend toward an association between the presence of circulating NETs and/or Cit-H3 and the comorbid conditions of SIRS or elevated cf-DNA concentration ($p = .1093$ and .3003, respectively), these were not statistically significant. Table 5 shows the results of multiple logistic regression analysis of factors associated with the presence of NETs and/or Cit-H3 and model selection. Two methods of multiple regression analysis, backward and forward regression, yielded similar models. Again, "the presence of bacteria in tracheal aspirate" was the only factor that was significantly related to the presence of NETs and/or Cit-H3 in the bloodstream; the odds ratio for aspiration was 5.750.

Discussion

A series of in vitro and animal experiments have uncovered a suppressive function of NETs against the dissemination of microorganisms in blood by mechanical trapping and by exploiting coagulant function to segregate these microorganisms within the circulation [28,29]. However, direct evidence remains scarce in living human systems. In this clinical study of blood smears, we attempted to identify morphologically the presence of NETs and Cit-H3 in the bloodstream of critically ill patients at the time of admission to the ICU and to characterize the factors associated with the presence of NETs and Cit-H3.

Among the 49 enrolled patients, immunofluorescence analysis revealed blood-borne NETs in five patients (10.2%), Cit-H3 in 11 patients (22.4%), and NETs and/or Cit-H3 in 15 patients (30.6%) (Table 2). These data replicate the results of our previous preliminary study in which NETs were present in patients in a critical condition [25] and show for the first time, to our knowledge, the presence of Cit-H3 in circulating blood cells. Cit-H3-positive cells possessed a multi-segmented nucleus, and most were immunoreactive for CD66b (Fig. 1), suggesting that citrullination of histone H3 occurred exclusively in neutrophils.

Table 1. Patient characteristics.

Variable	Value
No. of patients (M/F)	49 (29/20)
Age (years, median, IQR)	66.0 (52.5–76.0)
APACHE II score (median, IQR)	18.0 (12.5–21.5)
SOFA score (median, IQR)	5 (4–8)
No. of patients with SIRS	38 (77.6%)
The presence of bacteria in tracheal aspirate	22 (44.9%)
No. of survivors	36 (73.5%)
WBC (median, IQR)	10,900 (8215–14,915)

During the study period, 263 patients were admitted to the ICU of whom 49 were intubated and were included in this study. We excluded patients with cardiopulmonary arrest who could not be resuscitated on admission. IQR: interquartile range, APACHE: Acute Physiological And Chronic Health Evaluation, SOFA: Sequential Organ Failure Assessment, SIRS: systemic inflammatory response syndrome, WBC: white blood cell.
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Table 2. Diagnoses and the number of patients exhibiting neutrophil extracellular traps and citrullinated histone H3 in each diagnostic group.

Diagnosis	NET positive (n)	Cit-H3 positive (n)	NET and/or Cit-H3 positive (%)
Trauma (n = 7)	0	0	0/7 (0)
Infection (n = 14)	3	2	4/14 (28.6)
Resuscitated from cardiopulmonary arrest (n = 8)	2	3	5/8 (62.5)
Acute poisoning (n = 4)	0	1	1/4 (25.0)
Heart disease (n = 4)	0	0	0/4 (0)
Brain stroke (n = 8)	0	3	3/8 (37.5)
Heat stroke (n = 2)	0	1	1/2 (50.0)
Others (n = 2)	0	1	1/2 (50.0)
Total (n = 49)	5	11	15/49 (30.6)

In the blood smears surveyed in this study, we identified NETs in 5 patients and Cit-H3 in 11 patients. Both NETs and Cit-H3 were identified concurrently in one patient with infection. We found no NETs or Cit-H3-positive cells in samples from patients with trauma (0/7) or heart disease (0/4). NETs: neutrophil extracellular trap, Cit-H3: citrullinated histone H3.
doi:10.1371/journal.pone.0111755.t002

Citrullination of histone H3 is considered an important process in the release of NETs through decondensation of chromatin [14,22,23]. Interestingly, the occurrence ratio of Cit-H3 was twice that of NETs. In vitro experiments imply that a substantial period of time is necessary to expel NETs extracellularly after the initiation of cell death by a stress stimulus [12,30,31]. However, it is still not clear how much time is required in vivo for NETs to appear intravascularly. The number of patients who exhibited circulating NETs in this study was lower than anticipated. We collected blood samples on admission to the ICU, and the timing might have been too early to detect NETs after the onset of a

critical illness. The 11 Cit-H3-positive patients could be considered to have been in an early stage of NET formation. The change in the appearance of NETs and Cit-H3 during the course of hospitalization should be studied. If it can be shown clinically that Cit-H3 expression is followed by NET formation, it might be important to evaluate Cit-H3 expression in the blood upon admission to an ICU.

Table 2 shows that NETs and Cit-H3 were detected in patients with infection, resuscitation from CPA, acute poisoning, brain stroke, or heat stroke; surprisingly, we could not detect NETs or Cit-H3 in patients with trauma or heart disease. NETs are formed
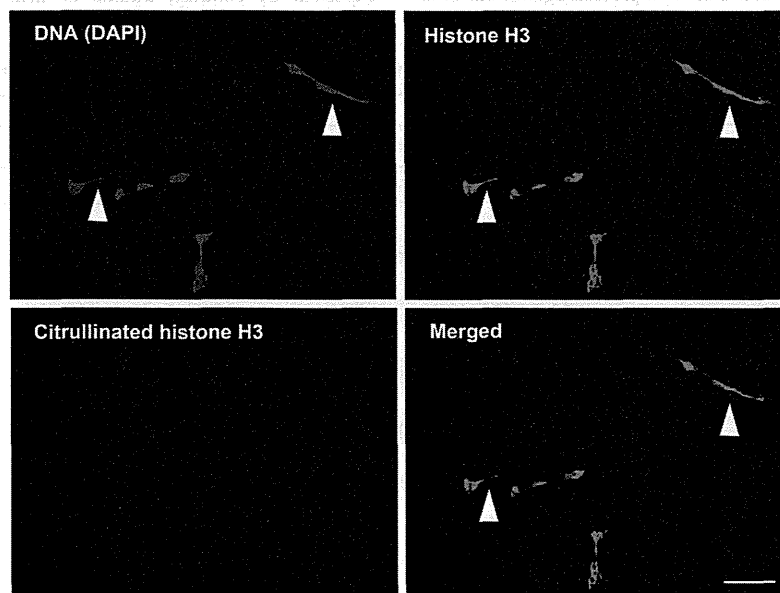

Figure 2. Representative images of immunofluorescence staining to detect neutrophil extracellular traps (NETs). NETs were visualized in the blood smear samples by immunocytochemistry and identified as extracellular string-like structures composed of chromatin (DNA and histone H3). NETs were present in the bloodstream of critically ill patients. Citrullination of histone H3 was not recognized in these images. In the blood smears surveyed in this study, we identified NETs in five patients (5/49, 10.2%). Blue, 4′,6-diamidino-2-phenylindole (DAPI); red, histone H3; green, citrullinated histone H3. Arrowheads indicate the double-stained areas containing NETs (Magnification ×400). Scale bar; 50 µm.
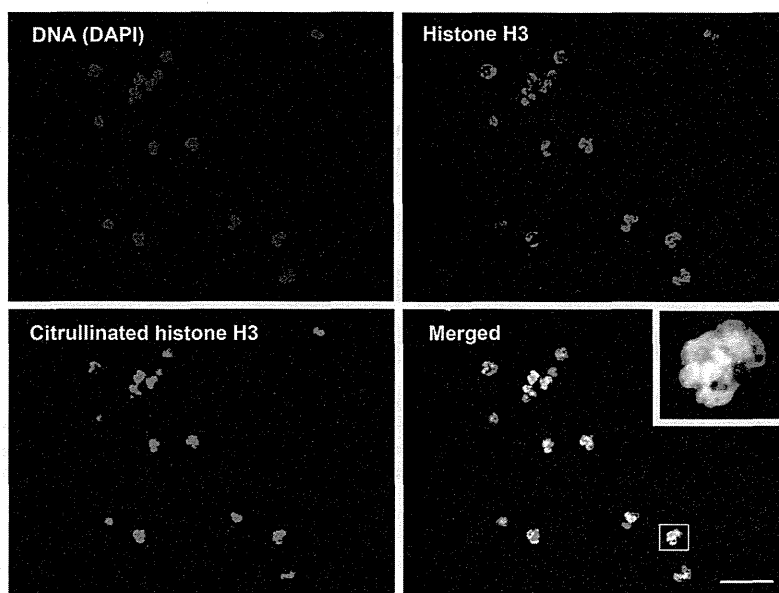doi:10.1371/journal.pone.0111755.g002

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Figure 3. Representative images of immunofluorescence staining to detect citrullinated histone H3 (Cit-H3). Citrullination of histone H3, which is a critical enzymatic process to produce NETs through decondensation of chromatin, was visualized in the blood smear samples using anti-citrullinated histone H3 antibody by immunohistochemistry. Cit-H3 was present in the bloodstream of critically ill patients. The inset in the merged image is the magnified image of a representative cell (white rectangle) expressing citrullinated histone H3 in the nucleus. Neutrophil extracellular traps are not recognized here. In the blood smears surveyed in this study, we identified Cit-H3 in 11 patients (11/49, 22.4%). Blue, 4',6-diamidino-2-phenylindole (DAPI); red, histone H3; green, citrullinated histone H3 (Magnification ×400). Scale bar; 50 µm.
doi:10.1371/journal.pone.0111755.g003

in response to various microorganisms and pathogens [14]. McDonald et al reported that NETs ensnare circulating bacteria and provide intravascular immunity that protects against bacterial dissemination during septic infection [29]. In this context, the presence of NETs and/or Cit-H3 in infected patients is to be expected. By contrast, trauma or heart disease patients were

Table 3. Comparison between patients positive and negative for neutrophil extracellular traps and/or citrullinated histone H3.

	NET and/or citrullinated histone H3		
	Positive	**Negative**	**p**
Number	15	34	
Age (years)	67.0 (49.0–78.0)	65.5 (56.8–75.3)	.8197
APACHE II score	20.0 (16.0–23.0)	17.5 (11.8–21.3)	.3171
SOFA score	6.0 (5.0–10.0)	5.0 (4.0–8.0)	.4062
Survivors (n)	10 (66.7%)	26 (76.5%)	.4737
SIRS patients (n)	14 (93.3%)	24 (70.6%)	.0786
The presence of bacteria in tracheal aspirate (n)	11 (73.3%)	11 (32.3%)	.0079
WBC count (/µl)	12,430 (8310.0–16510.0)	10,835 (8032.5–14307.5)	.5654
IL-8 (pg/mL)	57.6 (19.9–143.0)	65.3 (23.3–229.5)	.9136
TNF-α (pg/mL)	8.2 (6.2–21.6)	9.0 (4.8–16.3)	.9740
cf-DNA (ng/mL)	1038.3 (744.9–1329.7)	1072.7 (828.6–1770.7)	.6025
Lactate (mg/mL)	39 (11.0–71.0)	17.5 (12.0–56.3)	.5010
HMGB1 (ng/mL)	11.0 (6.8–21.5)	9.7 (5.9–16.3)	.5151

Among the factors evaluated to highlight the relation to the presence of NETs or Cit-H3 in the bloodstream, only "the presence of bacteria in tracheal aspirate" differed significantly between the NET- and/or Cit-H3-positive and -negative groups ($p<.01$). The other factors were not significantly related to the presence of NETs and/or Cit-H3. Continuous variables are presented as the median and IQR unless otherwise noted. The Wilcoxon rank-sum test and Pearson's chi-square test were used to compare two patient groups. NETs: neutrophil extracellular traps, Cit-H3: citrullinated histone H3, IQR: interquartile range, APACHE: Acute Physiological And Chronic Health Evaluation, SOFA: Sequential Organ Failure Assessment, SIRS: systemic inflammatory response syndrome, WBC: white blood cell, IL: interleukin, TNF: tumor necrosis factor, cf-DNA: circulating free DNA, HMGB1: high mobility group box-1.
doi:10.1371/journal.pone.0111755.t003

Table 4. Results of single logistic regression analysis.

Variable	p
The presence of bacteria in tracheal aspirate	.0112
SIRS	.1093
cf-DNA	.3003
Lactate	.5476
WBC count	.7862
IL-8	.7875
TNF-α	.8321
HMGB1	.9439

Logistic regression analysis was performed to identify the factors related to the presence of NET and Cit-H3 in the bloodstream. Only "the presence of bacteria in tracheal aspirate" (+) at the time of intubation was a significant factor associated with the presence of NET and Cit-H3 (p = .0112). NETs: neutrophil extracellular traps, Cit-H3: citrullinated histone H3, SIRS: systemic inflammatory response syndrome, cf-DNA: circulating free DNA, WBC: white blood cell, IL: interleukin, TNF: tumor necrosis factor, HMGB1: high mobility group box-1.
doi:10.1371/journal.pone.0111755.t004

transported to the hospital immediately after the onset of the condition, and there was no potential risk of infection on admission; this may explain why NETs and Cit-H3 were not detected in these patients.

Intriguingly, a high percentage (62.5%) of patients with CPA exhibited circulating NETs and/or Cit-H3. Acute poisoning, brain stroke, and heat stroke are clinical conditions that can cause disturbance of consciousness, which may induce aspiration. Adnet and Baud demonstrated that the risk of aspiration increases with the degree of unconsciousness (as measured by the Glasgow Coma Scale [GCS]) [32]. In the present study population, the GCS score on admission was significantly lower in the BTA (+) group than in the BTA (−) group (4 [IQR, 3–10.75] vs 13 [IQR, 7–14]; p<.01). Except for the infected patient group, the patients who exhibited NETs and/or Cit-H3 in their blood had a significantly lower GCS score on admission (p = .0418). We therefore investigated whether "the presence of bacteria in tracheal aspirate", which was represented as part of aspiration and as the presumable preclinical stage of manifested infection, was associated with the presence of NETs and/or Cit-H3, and found a significant association (odds ratio for aspiration, 5.750) (Tables 3–5). Bacteria drawn into the respiratory tract can induce epithelial injury, which provides an opportunity for bacterial translocation as well as leukocyte transmigration until completion of epithelial repair [33,34]. Concomitance of acid aspiration under impaired consciousness additionally enhances bacterial adherence to the epithelium [35]. Injured airway epithelium produces cytokines including IL-8 and alarmins such as HMGB1, both of which are representative inducers for NETs [36–39]. Next, bacteria and inflammatory

mediators infiltrating into the interstitial space secondary to epithelial injury will affect the endothelial integrity [40]. The presence of NETs in sputum following aspiration, a phenomenon that we reported previously [24], suggests breakdown of the epithelial barrier that is induced by local inflammation through direct contact between aspirated bacteria and epithelium or through activation of resident immune cells such as macrophages in the respiratory tract [41]. Such epithelial breakdown would allow influx of pathogens, pathogen-associated molecular patterns, cytokines, chemokines, and alarmins from the lumen of the respiratory tract into the circulation. These materials might stimulate the production of NETs intravenously to inhibit systemic invasion of bacteria. We assumed that NETs are induced in the respiratory tract to suppress bacterial dissemination leading to pneumonia and in the vessels to inhibit bacteremia against the invasion of bacteria into the blood and that even such colonization of bacteria in the respiratory tract could trigger citrullination of histone H3 to produce NETs in blood. Single logistic regression analyses of whether infection and/or BTA (+) were associated with the presence of NETs and/or Cit-H3 produced an odds ratio of 7.312 (Table S3). These results suggest that induction of NETs systemically through the citrullination of histone H3 in blood maybe an initial response for protection against bacterial dissemination from latent respiratory infection.

Some researchers consider cf-DNA to be equivalent to NETs in the blood [15,16]. However, our results showed that the occurrence rate of NETs and/or Cit-H3 was not significantly associated with cf-DNA concentration (p = .6025) (Table 3). Although the number of patients was different due to sample limitations, additional analysis by MPO-DNA ELISA (Data S1) was also performed. As a result, there was no difference in the values between the group positive for (0.076 [IQR, 0.067–0.100]; n = 8) and the group negative for NET and/or citrullinated histone H3 (0.078 [IQR, 0.070–0.111]; n = 26). We reported recently that in patients with an acute respiratory infection, NETs became fragmented during recovery from infection [24], suggesting that NETs should also be digested in the blood with time. Our method using blood smear samples cannot detect NETs that harbor inside vessels or that are already degraded, whereas the method based on MPO-DNA ELISA might also measure neutrophil DNA fragments derived from necrosis or apoptosis and cannot detect NETs that are not truncated from the cell body. We consider that at the early phase of critical illness, i.e., when the production of NETs is just starting, the morphological approach has an advantage in being able to detect NETs that are still anchored to the cell body, in conjunction with the merit that identification of citrullination of histone H3 is possible at a stage prior to the release of NETs.

HMGB1 is a nuclear protein present in the nucleus of all nucleated cells. HMGB1 binds to DNA and acts as an inflammatory mediator once it is released extracellularly [42,43]. In this study, HMGB1 was significantly higher in SIRS patients

Table 5. Results of multiple logistic regression analysis of factors associated with the presence of neutrophil extracellular traps and/or citrullinated histone H3.

	Coeff (β)	p	OR	Lower	Upper
"the presence of bacteria in tracheal aspirate"	0.875	0.011	5.750	1.583	24.755

Two methods of multiple regression analysis, backward and forward regression, yielded similar models. "The presence of bacteria in tracheal aspirate" was the only factor that was significantly related to the presence of neutrophil extracellular traps and/or citrullinated histone H3 in the bloodstream. The odds ratio for aspiration was 5.750. Coeff (β): coefficient; OR: odds ratio, Lower: lower level of 95% confidence interval, Upper: upper level of 95% confidence interval.
doi:10.1371/journal.pone.0111755.t005

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than in non-SIRS patients (Table S2). Unexpectedly, however, HMGB1 was not a significant factor associated with the presence of NETs and/or Cit-H3 (Tables 3–5). NETs contain HMGB1 [44], and one possibility is that HMGB1 binding to NETs is not reflected in the amount of circulating HMGB1 measured by ELISA.

Although IL-8 and TNF-α are considered stimulatory factors that induce NET formation [14,39,45], they were not associated with the presence of NETs and/or Cit-H3 in this study (Tables 3–5). This negative result suggest the presence of an unknown complex regulatory mechanism for the production of NETs in vivo.

As limitations of this study, first, the sample size was small, and the patients were very heterogeneous. Second, we evaluated the presence of NETs and Cit-H3 and the associated factors in the bloodstream of critically ill patients only at admission. It should be investigated in the future how NETs are processed after the induction of NETosis in the circulation. It is presumable that NETs could be degraded by DNase, and the fragments would contribute partially to the formation of cf-DNA. Third, we did not rigorously quantify the amount of NETs and Cit-H3. The possibility of the degradation of NETs and the difficulty in detecting NETs, which are anchored in the vessels, might lead to underestimation of the presence of NETs in our method using blood smear samples. Further study is required to establish finer methods of quantification. We hope that future elucidation of the biological significance of NETs will lead to new strategies to treat critical illness by monitoring NET formation in blood.

Conclusions

The presence of NETs and Cit-H3 were identified immunocytochemically in the bloodstream of a subset of critically ill patients. "The presence of bacteria in tracheal aspirate" may be one important factor related to the presence of circulating NETs. NETs may play a pivotal role in biological defense in the bloodstream of infected and potentially infected patients.

Supporting Information

Figure S1 Representative images of immunostaining of isolated neutrophils that underwent drying and freezing steps before fixation. We tried to evaluate the influence of drying and freezing steps preceding paraformaldehyde fixation on the induction of NETs or citrullination of histone H3 in smear samples. For this, neutrophils separated by density gradient centrifugation from whole blood of a healthy donor were smeared on glass slides, dried, and frozen before fixation. At least through this method, the presence of NETs or citrullinated histone H3 was not identified in immunostaining. Blue, Hoechst 33342; Red, histone H3; Green, citrullinated histone H3 (left panels) or neutrophil elastase (right panels) (Magnification ×400). Scale bar; 50 μm.
(TIF)

Figure S2 Representative images of immunostaining for the negative control study using isotype control antibodies. To ensure accuracy for the immunoreactivity of primary antibodies against blood smear samples, whole mouse and rabbit IgG were used instead of primary antibodies in the immunostaining procedure. This control study resulted in negative signals for histone H3 and citrullinated histone H3. Blue, 4′,6-

diamidino-2-phenylindole (DAPI); Red, histone H3; Green, citrullinated histone H3. (Magnification ×200). Scale bar; 50 μm. (TIF)

Figure S3 Representative images of immunostaining to detect citrullinated histone H3 (left panels) and neutrophil extracellular traps (NETs) (right panels) in the neutrophils from a healthy donor stimulated by phorbol myristate acetate. Neutrophils were isolated by density gradient centrifugation from the whole blood of a healthy donor and stimulated by phorbol myristate acetate. Citrullinated histone H3 and NETs were detected by immunohistochemistry using the same antibodies that were used against the smear samples collected from the critically ill patients. Blue, Hoechst 33342; Red, histone H3; Green, citrullinated histone H3 (left panels) or neutrophil elastase (right panels). (Magnification ×400). Scale bar; 50 μm.
(TIF)

Figure S4 Representative images of immunostaining to detect neutrophil extracellular traps (NETs) in the blood smear from a critically ill patient. The presence of circulating NETs was confirmed by immunohistochemistry using anti-neutrophil elastase antibody. String-like structures extending from the cell body (arrowheads) were composed of DNA and histone, and they contained neutrophil elastase. Blue, 4′,6-diamidino-2-phenylindole (DAPI); Red, histone H1; Green, Neutrophil elastase. (Magnification ×400). Scale bar; 50 μm.
(TIF)

Figure S5 Diff-Quik staining of a blood smear sample from the critically ill patient. Diff-Quik staining confirmed a subpopulation of cells other than neutrophils. (Magnification ×400). Scale bar; 50 μm.
(TIF)

Table S1 Comparison between patients presenting with and without "the presence of bacteria in tracheal aspirate". In patients classified into two groups based on the presence or absence of bacteria in tracheal aspirate, the rate of occurrence of NETs and/or Cit-H3 was significantly higher in "the presence of bacteria in tracheal aspirate" group (11/22, 50.0%) than in "the absence of bacteria in tracheal aspirate" group (4/27, 14.8%) ($p<.01$). Continuous variables are presented as the median and IQR unless otherwise noted. The Wilcoxon rank-sum test and Pearson's chi-square test were used to compare the two patient groups. NETs: neutrophil extracellular traps, Cit-H3: citrullinated histone H3, IQR: interquartile range, APACHE: Acute Physiological And Chronic Health Evaluation, SOFA: Sequential Organ Failure Assessment, SIRS: systemic inflammatory response syndrome, WBC: white blood cell, IL: interleukin, TNF: tumor necrosis factor, cf-DNA: circulating free DNA, HMGB1: high mobility group box-1.
(DOCX)

Table S2 Comparison between patients with and without systemic inflammatory response syndrome. In patients with SIRS on admission, there was a trend toward greater expression of NETs and/or Cit-H3 ($p = .079$). Continuous variables are presented as the median and IQR unless otherwise noted. The Wilcoxon rank-sum test and Pearson's chi-square test were used to compare the two patient groups. NETs: neutrophil extracellular traps, Cit-H3: citrullinated histone H3, IQR: interquartile range, APACHE: Acute Physiological And Chronic Health Evaluation, SOFA: Sequential Organ Failure Assessment, SIRS: systemic inflammatory response syndrome,

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WBC: white blood cell, IL: interleukin, TNF: tumor necrosis factor, cf-DNA: circulating free DNA, HMGB1: high mobility group box-1.
(DOCX)

Table S3 Results of single logistic regression analysis of factors associated with the presence of neutrophil extracellular traps and/or citrullinated histone H3 according to the presence of infection and/or "the presence of bacteria in tracheal aspirate". Single logistic regression analyses of whether infection and/or "the presence of bacteria in tracheal aspirate" were associated with the presence of NETs and/or Cit-H3 produced an odds ratio of 7.312. Coeff (β):

coefficient, OR: odds ratio, Lower: lower level of 95% confidence interval, Upper: upper level of 95% confidence interval.
(DOCX)

Data S1 MPO-DNA ELISA.
(DOCX)

Author Contributions

Conceived and designed the experiments: TH SH NM TI. Performed the experiments: TH SH NM TI HH NY. Analyzed the data: TH SH NM TI MS OT NY KY YA KO TS KT. Contributed reagents/materials/analysis tools: TH SH HH NY. Wrote the paper: TH SH NM.

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Effectiveness of a simplified cardiopulmonary resuscitation training program for the non-medical staff of a university hospital

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Abstract

Background: The 2010 Consensus on Science and Treatment Recommendations Statement recommended that short video/computer self-instruction courses, with minimal or no instructor coaching, combined with hands-on practice can be considered an effective alternative to instructor-led basic life support courses. The purpose of this study was to examine the effectiveness of a simplified cardiopulmonary resuscitation (CPR) training program for non-medical staff working at a university hospital.

Methods: Before and immediately after a 45-min CPR training program consisting of instruction on chest compression and automated external defibrillator (AED) use with a personal training manikin, CPR skills were automatically recorded and evaluated. Participants' attitudes towards CPR were evaluated by a questionnaire survey.

Results: From September 2011 through March 2013, 161 participants attended the program. We evaluated chest compression technique in 109 of these participants. The number of chest compressions delivered after the program *versus* that before was significantly greater (110.8 ± 13.0/min vs 94.2 ± 27.4/min, $p < 0.0001$), interruption of chest compressions was significantly shorter (0.05 ± 0.34 sec/30 sec vs 0.89 ± 3.52 sec/30 sec, $p < 0.05$), mean depth of chest compressions was significantly greater (57.6 ± 6.8 mm vs 52.2 ± 9.4 mm, $p < 0.0001$), and the proportion of incomplete chest compressions of <5 cm among all chest compressions was significantly decreased ($8.9 \pm 23.2\%$ vs $38.6 \pm 42.9\%$, $p < 0.0001$). Of the 159 participants who responded to the questionnaire survey after the program, the proportion of participants who answered 'I can check for a response,' 'I can perform chest compressions,' and 'I can absolutely or I think I can use an AED' increased *versus* that before the program (81.8% vs 19.5%, 77.4% vs 10.1%, 84.3% vs 23.3%, respectively).

Conclusions: A 45-min simplified CPR training program on chest compression and AED use improved CPR quality and the attitude towards CPR and AED use of non-medical staff of a university hospital.

Background

Bystander-initiated cardiopulmonary resuscitation (CPR) and the automated external defibrillator (AED) have major roles in the 'chain of survival' for both out-of-hospital and in-hospital cardiac arrest [1,2]. The effectiveness of the rapid response system for in-hospital cardiac arrest or critically ill patients has been reported [3,4], and in our hospital, the rapid response system was introduced in 2001. During an emergency, the rapid response team (emergency medicine doctors and nurses) are paged to rush to the stricken patient. However, education for the non-medical staff working at medical institutions, who could potentially be first responders and could activate the system, has not been established.

The 2010 Consensus on Science and Treatment Recommendations (CoSTR) Statement recommended that training should aim to ensure that learners acquire and retain the skills and knowledge that will enable them to act correctly during actual cardiac arrests, and short video/computer self-instruction courses, with minimal or no instructor coaching, combined with hands-on practice can be considered as an effective alternative to

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instructor-led basic life support (BLS) courses [5]. In September 2010, we introduced a 45-min simplified CPR training program consisting of instruction on chest compression and AED use with a personal training manikin for the non-medical staff working at our university hospital. From September 2011, the quality of CPR skills was recorded *via* a CPR skill report system, and a questionnaire survey on the participants' attitudes towards CPR and AED use was conducted before and immediately after this training program. The purpose of this study was to examine the effectiveness of a simplified CPR training program for the non-medical staff working at a university hospital.

Methods

Study design

This was a prospective observational study that was approved by the Ethics Committee of Osaka University Graduate School of Medicine. The institutional review board waived the need for informed consent. We surveyed participants who attended this CPR training program from September 2011 through March 2013. Those eligible to participate in this program were non-medical staff working at our university hospital.

Simplified CPR training program

The simplified CPR training program consisted of instructions and practice on chest compressions and AED use with a personal training manikin, and the total time of this program was 45 minutes. We used the CPR Training Box APPA-KUN® obtained from the non-profit organisation Osaka Life Support Association, Osaka, Japan, as the personal training manikin (Figure 1A). Doctors and nurses who were instructors of the Immediate Cardiac Life Support (ICLS) course certified by the Japanese Association for Acute Medicine (JAAM) or instructors with equivalent qualifications and who were specially trained for

this program instructed the participants. The instructor/participant ratio was 1:10–20. A photo of this training program in action is shown in Figure 2. The training program was DVD-based and could be held using a small number of instructors (at least one instructor was required). Table 1 shows the time schedule of this training program, which consisted of an opening speech; introduction containing a check of the participants' knowledge about 'check for a response', 'chest compressions', and 'AED use'; explanation of the rapid response system; simulation of an in-hospital resuscitation by DVD; practice on chest compression and AED use with the personal training manikin; and a question and answer session. We standardised the contents of the training program by using the DVD presentation.

Evaluation of CPR skills

Before and immediately after the 45-min CPR training, CPR skills were recorded *via* the CPR skill report system APPA-KUN Pro® (Alexon, Osaka, Japan) (Figure 1B). This CPR evaluation system automatically records the number of chest compressions, interruption of chest compressions, and depth of chest compressions. The evaluation of CPR skills was performed on the participants, whose cooperation was voluntary, and the time for evaluation of CPR skills was 30 seconds due to restrictions of time and the number of CPR skill report systems available. We evaluated the participants' CPR skill at each training program attended, as well as for all programs attended.

Questionnaire survey evaluating participants' attitudes towards CPR and AED use

Before and immediately after the 45-min CPR training, participants' attitudes towards CPR and AED use were evaluated by a questionnaire survey. The question items included 'Can you check for a response?', 'Can you
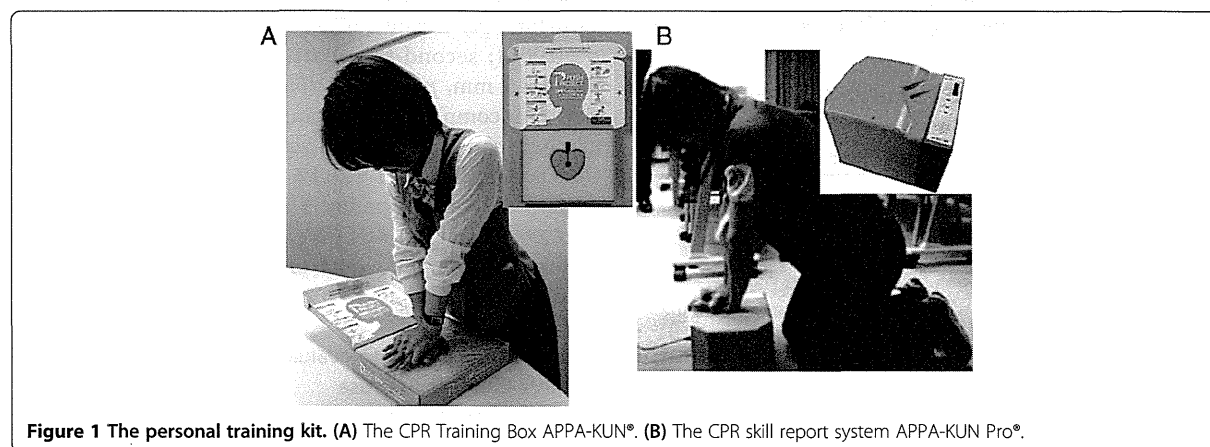

Figure 1 The personal training kit. (A) The CPR Training Box APPA-KUN®. **(B)** The CPR skill report system APPA-KUN Pro®.
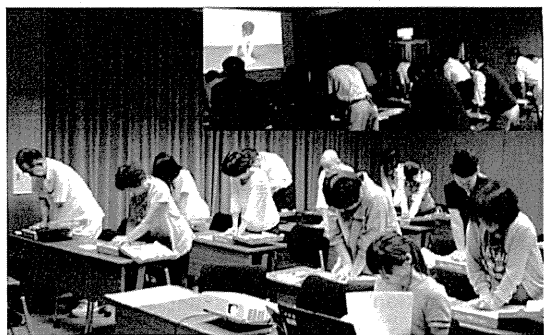
Figure 2 The classroom scene of the simplified cardiopulmonary resuscitation training program with personal training kit.

perform chest compression?', and 'Can you use an AED?'. The questionnaire survey was given to all participants, and replies were anonymous. The participants provided one answer for each multiple choice question.

Statistical analysis

All data are represented as mean ± standard deviation (SD). The Wilcoxon signed rank test was used to compare the differences between before and immediately after training, and the Wilcoxon rank sum test was used to compare the differences between the first-time and the second-time participants in the evaluation of CPR skills. A value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed with JMP 9.0.2 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Evaluation of CPR skills

From September 2011 through March 2013, 161 participants attended the program, and we evaluated the chest compression technique of 109 participants due to restrictions of time and the number of CPR skill report systems available. The study group comprised 44 men and 65 women with a mean age ± SD of 42.2 ± 14.7 years.

Table 1 Time schedule of the simplified cardiopulmonary resuscitation training program

Training schedule	Device used	Time (min)
Welcome		2
Introduction (check of knowledge)	DVD	6
Rapid response system and simulation of an in-hospital resuscitation	DVD	6
Chest compression and AED use	Practice with a personal training manikin	26
Question and answer session		5
Total		45

Among the 109 participants, 57 were the first-time participants of CPR training, 48 were second-time participants, and 4 were participating for the third time or more. In the analysis of the 109 participants, the number of chest compressions was significantly greater (110.8 ± 13.0/min vs 94.2 ± 27.4/min, $p < 0.0001$) (Figure 3A), the interruption of chest compressions was significantly shorter (0.05 ± 0.34 sec/30 sec vs 0.89 ± 3.52 sec/30 sec, $p < 0.05$) (Figure 3B), the mean depth of chest compressions was significantly greater (57.6 ± 6.8 mm vs 52.2 ± 9.4 mm, $p < 0.0001$) (Figure 3C), and the proportion of incomplete chest compressions of <5 cm among all chest compressions was significantly decreased (8.9 ± 23.2% vs 38.6 ± 42.9%, $p < 0.0001$) (Figure 3D) after the program *versus* before the program.

We also compared CPR skill between the first and second attendance. The interval of attendance was 12.7 ± 4.3 months. Before the program, the number of chest compressions in the second-time participants was significantly greater (100.9 ± 28.4/min vs 87.7 ± 26.0/min, $p < 0.05$) (Figure 4A), and the interruption of chest compressions was significantly shorter (0.08 ± 0.58 sec/30 sec vs 1.62 ± 4.7 sec/30 sec, $p < 0.05$) (Figure 4B), compared with these values in the first-time participants. There were no significant differences between the two groups in mean depth of chest compressions (52.6 ± 8.6 mm vs 51.2 ± 10.1 mm, $p = 0.42$) (Figure 4C) and in the proportion of incomplete chest compressions of <5 cm among all chest compressions (35.9 ± 44.7% vs 43.5 ± 41.6%, $p = 0.32$) (Figure 4D). After the program as compared with before, the number of chest compressions was significantly greater (first-time participants: 109.5 ± 13.7/min vs 87.7 ± 26.0/min, $p < 0.0001$; second-time participants: 111.8 ± 12.5/min vs 100.9 ± 28.4/min, $p < 0.05$) (Figure 4A), the interruption of chest compressions was shorter (first-time participants: 0.09 ± 0.47 sec/30 sec vs 1.62 ± 4.7 sec/30 sec, $p < 0.05$; second-time participants: 0.0 ± 0.0 sec/30 sec vs 0.08 ± 0.58 sec/30 sec, $p = 1.0$) (Figure 4B), the mean depth of chest compressions was significantly greater (first-time participants: 57.9 ± 8.1 mm vs 51.2 ± 10.1 mm, $p < 0.0001$; second-time participants; 57.1 ± 5.1 mm vs 52.6 ± 8.6 mm, $p < 0.0001$) (Figure 4C), and the proportion of incomplete chest compressions of <5 cm among all chest compressions was significantly decreased (first-time participants: 10.1 ± 26.1% vs 43.5 ± 41.6%, $p < 0.0001$; second-time participants: 8.2 ± 20.3% vs 35.9 ± 44.7%, $p < 0.0001$) (Figure 4D). We excluded the participants who attended a third time or more because of the low number of participants.

Questionnaire survey to evaluate participants' attitudes towards CPR and AED use

Responses to the questionnaire survey were obtained from 159 (98.8%) of the 161 participants. The responder

Figure 3 Evaluation of cardiopulmonary resuscitation (CPR) skills for all participants before and immediately after the simplified CPR training program. (A) Number of chest compressions, **(B)** time chest compressions were interrupted, **(C)** mean depth of chest compressions, and **(D)** proportion of incomplete chest compressions of <5 cm among all chest compressions.
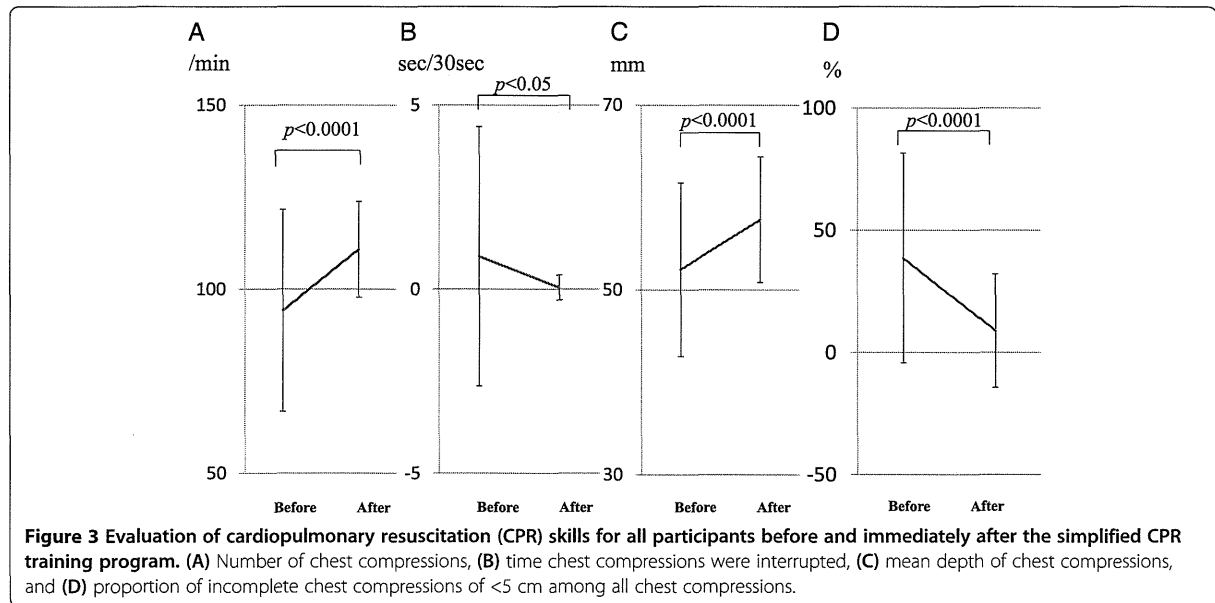
group comprised 56 men and 103 women with a mean age ± SD of 42.5 ± 14.2 years, with 95 first-time, 59 second-time, and 5 third-time or more participants.

After the program as compared with before, the proportion of participants who answered 'I can check for a response', 'I can perform chest compressions', and 'I

absolutely can or I think I can use an AED' increased (81.8% vs 19.5%, 77.4% vs 10.1%, and 84.3% vs 23.3%, respectively) (Table 2).

In the second-time participants, the proportion of participants who answered 'I can't or shouldn't check for a response' was smaller (2 [3.4%] vs 23 [24.2%]), who

Figure 4 Evaluation of cardiopulmonary resuscitation (CPR) skills according to first- and second-time attendance of the participants.
(A) Number of chest compressions, **(B)** time chest compressions were interrupted, **(C)** mean depth of chest compressions, and **(D)** proportion of incomplete chest compressions of <5 cm among all chest compressions.
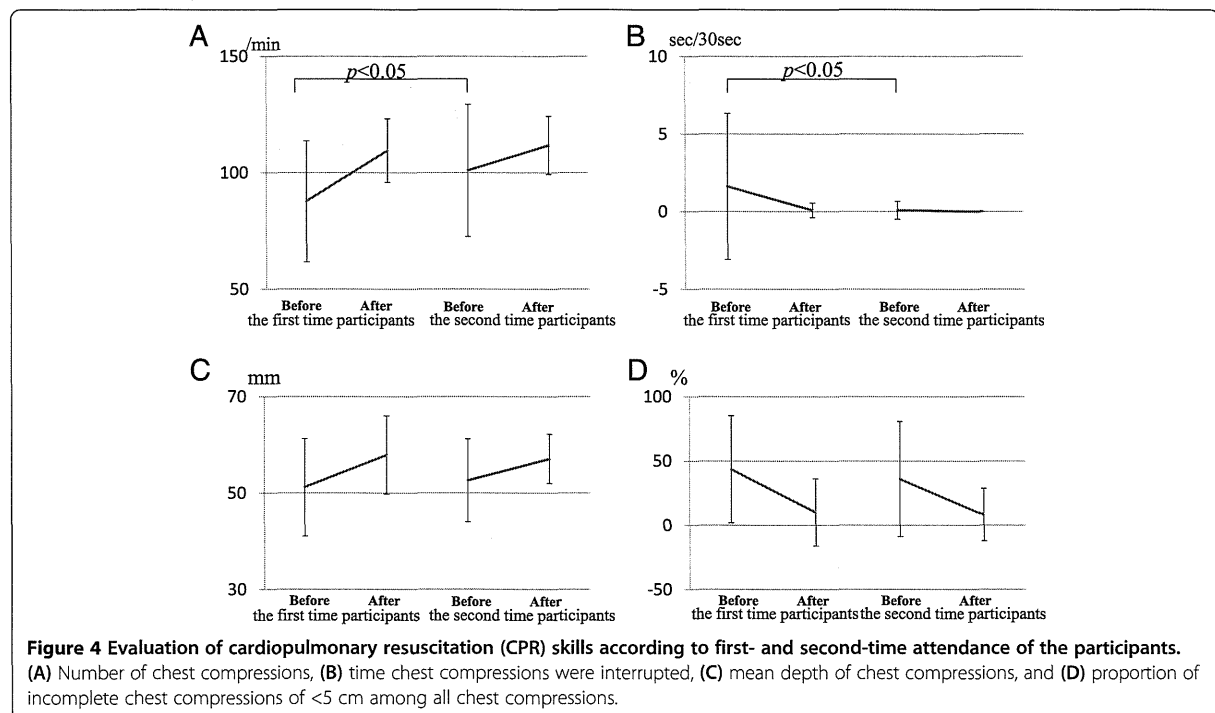
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Table 2 Results of the questionnaire survey to evaluate participants' attitudes towards cardiopulmonary resuscitation and automated external defibrillator (AED) use

	Total (N = 159)		First-time participants N = 95)		Second-time participants(N = 59)	
	Before	After	Before	After	Before	After
Q. 1 Can you check for a response?						
I can.	31 (19.5%)	130 (81.8%)	14 (14.7%)	80 (84.2%)	15 (25.4%)	47 (79.7%)
I don't know if I can.	103 (64.8%)	29 (18.2%)	58 (61.1%)	15 (15.8%)	42 (71.2%)	12 (20.3%)
I can't.	21 (13.2%)	0 (0%)	20 (21.1%)	0 (0%)	1 (1.7%)	0 (0%)
I shouldn't. More skillful people should.	4 (2.5%)	0 (0%)	3 (3.2%)	0 (0%)	1 (1.7%)	0 (0%)
Q. 2 Can you perform chest compression?						
I can.	16 (10.1%)	123 (77.4%)	8 (8.4%)	75 (78.9%)	7 (11.8%)	46 (78.0%)
I don't know if I can.	88 (55.3%)	36 (22.6%)	38 (40.0%)	20 (21.1%)	46 (78.0%)	13 (22.0%)
I can't.	49 (30.8%)	0 (0%)	43 (45.3%)	0 (0%)	6 (10.2%)	0 (0%)
I shouldn't. More skillful people should.	6 (3.8%)	0 (0%)	6 (6.3%)	0 (0%)	0 (0%)	0 (0%)
Q. 3 Can you use an AED?						
I absolutely can.	4 (2.5%)	27 (17.0%)	2 (2.1%)	17 (17.9%)	1 (1.7%)	9 (15.3%)
I think I can.	33 (20.8%)	107 (67.3%)	14 (14.7%)	67 (70.5%)	18 (30.5%)	38 (64.4%)
I don't know if I can.	58 (36.5%)	19 (11.9%)	31 (32.6%)	7 (7.4%)	25 (42.4%)	10 (16.9%)
I think I can't.	47 (29.6%)	4 (2.5%)	34 (35.8%)	3 (3.2%)	12 (20.3%)	1 (1.7%)
I absolutely can't.	17 (10.7%)	2 (1.3%)	14 (14.7%)	1 (1.1%)	3 (5.1%)	1 (1.7%)

answered 'I can't or shouldn't perform chest compression' was smaller (6 [10.2%] vs 49 [51.6%]), and who answered 'I absolutely can't or I think I can't use an AED' was smaller (15 [25.4%] vs 48 [50.5%]) than that of the first-time participants. After the program as compared with before, the proportion of participants who answered 'I can check for a response' was greater (first-time participants: 80 [84.2%] vs 14 [14.7%]; second-time participants: 47 [79.7%] vs 15 [25.4%]), who answered 'I can perform chest compressions' was greater (first-time participants: 75 [78.9%] vs 8 [8.4%]; second-time participants: 46 [78.0%] vs 7 [11.8%]), and who answered 'I absolutely can or I think I can use an AED' was greater (first-time participants: 84 [88.4%] vs 16 [16.8%]; second-time participants: 47 [79.7%] vs 19 [32.2%]) (Table 2).

Discussion

In this study, we showed the effectiveness of a simplified 45-min CPR training program for non-medical staff working at a university hospital that improved both the quality of CPR and the attitude of the staff towards CPR and AED use.

CPR and AED use by bystanders are very important in the 'chain of survival' for both out-of-hospital and in-hospital cardiac arrests to improve patient survival [1,2]. It was reported that survival to hospital discharge is still about 15% to 20% after in-hospital-cardiac arrest [6]. The non-medical staff working at a large hospital potentially can be the first responders for patients requiring CPR in most settings. To improve the 'chain of survival',

especially in the first three links of the chain and to quickly activate the rapid response team, an adequate educational program is needed for these personnel.

Recently, animal and clinical research suggested that bystander-initiated cardiac-only resuscitation is at least as effective as conventional CPR for ventricular fibrillation (VF) or short periods of untreated arrest [7–10]. In addition, it was reported that cardiac-only resuscitation without mouth-to-mouth ventilation was easier to learn and perform and made it possible for the general public to perform a greater number of appropriate chest compressions than with the conventional CPR program [11,12]. Therefore, we introduced a 45-min simplified CPR training program consisting of instruction and practice in chest compression and AED use with a personal training manikin for the non-medical staff working at our university hospital because we needed to educate a number of these personnel in a short time.

In this study, we successfully demonstrated an improvement in the quality of CPR after the simplified CPR training course (Figure 3). The 2010 CoSTR Statement emphasised the need for improving the quality of CPR to increase patient survival after cardiac arrest [5]. Christenson et al. reported that the chest compression fraction appears to be an important determinant of survival from cardiac arrest [13]. It was also reported that shallower chest compressions correlated significantly with a decrease in successful defibrillation [14,15]. Now, the rescuer should give chest compressions to a depth of at least 5 cm and at a rate of at least 100 times per

minute, allow full chest recoil after each compression, and minimise interruptions in chest compression [5]. The improvement in the quality of CPR seen in the participants after the simplified CPR training may lead to an improvement in the prognosis of patient suffering in-hospital cardiac arrest in our hospital. Further analysis of serial in-hospital cardiac arrest statistics would prove this hypothesis.

In this study, we conducted a questionnaire survey to evaluate participants' attitudes towards CPR and AED use and demonstrated an improvement in their attitudes after the training (Table 2). Our result was consistent with that of a previous report that indicated that in an actual emergency setting, the participants of CPR training were more likely to perform CPR than those without the experience of CPR training [16]. However, Dwyer reported that even if the participants answered that they were confident that they could initiate CPR after CPR training, they could not perform CPR adequately in an actual emergency situation [17]. Therefore, further research as to whether the result in this study will lead to an increase in the initiation of CPR in an actual emergency situation is needed.

We found that the quality of CPR and the participants' attitude towards CPR and AED use were better in the second-time participants than in the first-time participants before CPR training (Figure 4, Table 2). BLS and advanced cardiac life support (ACLS) knowledge and skills can deteriorate in as little as 3 to six months [5,18,19]. Therefore, more frequent assessments or refresher training is recommended to maintain knowledge and skills [5]. In the present study, the interval of attendance from the last CPR training was 12.7 ± 4.3 months, and even though the CPR skills and attitude towards CPR of the second-time participants were better than those without experience of previous CPR training, they were not still sufficient (Figure 4D). As such, repeated attendance at an interval of less than 1 year would be desirable. The optimal interval between CPR training programs to maintain the quality of CPR and attitude towards CPR and AED use requires further clarification.

The 45-min time of this training program was shorter than that of conventional BLS or ACLS courses, and this training could be conducted by at least one instructor. Therefore, less burdens on time and expense exist for either the participants or the instructors. Because our program improved the quality of CPR and attitude towards CPR and AED use of the participants, this program could be considered not only in medical but also in non-medical institutions when implementing CPR and AED use.

As limitations of the present study, first, due to restrictions of time and the number of available CPR skill report systems, the evaluation of CPR skills was performed for only 30 seconds on 109 participants in whom cooperation was voluntary. Second, we did not test the practical skill of the participants on the AED due to restrictions of time. Further study is needed to evaluate the appropriate use of an AED after the training program. Third, there is no data on longer-term retention of skills. Forth, whether the participants of this CPR training could actually perform CPR in an emergency situation is unknown.

Conclusion

A simplified 45-min CPR training program combining instruction and practice in chest compression and AED use improved the quality of CPR and the attitude towards CPR and AED use of the non-medical staff working at a university hospital. Further study to reveal the optimal interval for conducting the CPR training program to maintain the quality of CPR and a positive attitude towards CPR and AED use is needed.

Authors' contributions
TH, TI, and TS designed the study. TH, HM, TS, and KY collected and generated the data. TH wrote the first draft. TH, TI, HO, KY, TM, YF and TS analyzed the data and helped to draft the manuscript. All of the authors read and approved the final manuscript.

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Model based on skew normal distribution for square contingency tables with ordinal categories

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ABSTRACT

For the analysis of square contingency tables with ordinal categories, Tahata, Yamamoto and Tomizawa (2009) considered the normal distribution type symmetry model, which may be appropriate if it is reasonable to assume an underlying bivariate normal distribution with equal marginal variances. The present paper proposes a new model which may be appropriate for a square ordinal table if it is reasonable to assume an underlying bivariate skew normal distribution with equal marginal variances. Simulations are used to investigate the fitting of new model for bivariate skew normal distribution. The decayed teeth data are analyzed by using the new model.

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1. Introduction

Consider an $r \times r$ square contingency table with the same ordinal row and column classifications. Let p_{ij} denote the probability that an observation will fall in the (i, j)th cell of the table $(i = 1, \ldots, r; j = 1, \ldots, r)$.

Agresti (1983) proposed the linear diagonals-parameter symmetry model, namely LDPS, as follows:

$$\frac{p_{ij}}{p_{ji}} = \theta^{j-i} \quad (i < j).$$

A special case of this model obtained by putting $\theta = 1$ is the symmetry model (see, e.g., Bowker, 1948; Bishop et al., 1975, p. 282).

Let the random variable $X = (U, V)^T$ be distributed according to the bivariate normal distribution with means $E(U) = \mu_1$ and $E(V) = \mu_2$, variances $\text{Var}(U) = \text{Var}(V) = \sigma^2$, and correlation $\text{Corr}(U, V) = \rho$. The density function is expressed as

$$f(u, v) = \frac{1}{2\pi\sigma^2\sqrt{1 - \rho^2}} \exp\left[-\frac{1}{2\sigma^2(1 - \rho^2)} \left((u - \mu_1)^2 - 2\rho(u - \mu_1)(v - \mu_2) + (v - \mu_2)^2\right) \right] \quad (u, v \in R).$$

Then it satisfies

$$\frac{f(u, v)}{f(v, u)} = \exp\left(\frac{(v - u)(\mu_2 - \mu_1)}{(1 - \rho)\sigma^2} \right) \quad (u < v).$$

Agresti (1983) described that $f(u, v)/f(v, u)$ has the form θ^{v-u} for some constant θ, and hence the LDPS model may be appropriate for a square ordinal table if it is reasonable to assume an underlying bivariate normal distribution with equal marginal variances (see also Tomizawa, 1991; Yamamoto et al., 2007; Yamamoto et al., 2008; Tahata and Tomizawa, 2010).

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In addition, the normal density $f(u, v)$ can be expressed as

$$f(u, v) = c a_1^{(u-v)^2} a_2^{u-v} b_1^{(u+v)^2} b_2^{u+v},$$

where

$$c = \frac{1}{2\pi\sigma^2\sqrt{1 - \rho^2}} \exp\left[-\frac{(\mu_1 - \mu_2)^2}{4\sigma^2(1 - \rho)} - \frac{(\mu_1 + \mu_2)^2}{4\sigma^2(1 + \rho)}\right],$$

$$a_1 = \exp\left(\frac{-1}{4\sigma^2(1 - \rho)}\right),$$

$$a_2 = \exp\left(\frac{\mu_1 - \mu_2}{2\sigma^2(1 - \rho)}\right),$$

$$b_1 = \exp\left(\frac{-1}{4\sigma^2(1 + \rho)}\right),$$

$$b_2 = \exp\left(\frac{\mu_1 + \mu_2}{2\sigma^2(1 + \rho)}\right).$$

Then, the normal distribution type symmetry (NDS) model was proposed by Tahata et al. (2009) as follows:

$$p_{ij} = \xi \alpha_1^{(i-j)^2} \alpha_2^{i-j} \beta_1^{(i+j)^2} \beta_2^{i+j} \quad (i = 1, \ldots, r; j = 1, \ldots, r).$$

This model is a special case of the LDPS model. Tahata et al. (2009) pointed out that the $\{p_{ij}\}$ has a similar structure to the bivariate normal density with equal marginal variances, and hence the NDS model may also be appropriate for a square ordinal table if it is reasonable to assume an underlying bivariate normal distribution with equal marginal variances.

The skew normal distribution (e.g., Azzalini and Dalla Valle, 1996) is well known to be an extended distribution of the normal distribution. A standard bivariate skew normal density function $g_2(\boldsymbol{u})$ is given as

$$g_2(\boldsymbol{u}) = 2\phi_2(\boldsymbol{u})\Phi(\boldsymbol{\gamma}'\boldsymbol{u}) \quad (\boldsymbol{u} \in R^2), \tag{1}$$

where $\phi_2(\cdot)$ and $\Phi(\cdot)$ denote, respectively, the probability density function of the $N_2(\boldsymbol{0}, I_2)$ distribution and cumulative distribution function of the standard normal distribution, and $\boldsymbol{\gamma} \in R^2$ is the skewness parameter (see Baghfalaki and Ganjali, 2011). More generally, for an $N_2(\boldsymbol{\mu}, \Omega)$ distribution, we denote the equivalent functions by $\phi_2(\cdot; \boldsymbol{\mu}, \Omega)$. As a location-scale extension of (1), the bivariate skew normal density $h(\boldsymbol{v})$ is given as

$$h(\boldsymbol{v}) = 2\phi_2(\boldsymbol{v}; \boldsymbol{\mu}, \Omega)\Phi(\boldsymbol{\gamma}'\Omega^{-1/2}(\boldsymbol{v} - \boldsymbol{\mu})) \quad (\boldsymbol{v} \in R^2), \tag{2}$$

with the mean vector $\boldsymbol{\mu}$, the covariance matrix Ω, and the skew vector $\boldsymbol{\gamma}$, where

$$\boldsymbol{\mu} = \begin{pmatrix} \mu_1 \\ \mu_2 \end{pmatrix}, \qquad \Omega = \sigma^2 \begin{pmatrix} 1 & \rho \\ \rho & 1 \end{pmatrix}, \qquad \boldsymbol{\gamma} = \begin{pmatrix} \gamma_1 \\ \gamma_2 \end{pmatrix}.$$

When $\boldsymbol{\gamma} = \boldsymbol{0}$, the density (2) is equivalent to the density of the $N_2(\boldsymbol{\mu}, \Omega)$ distribution. Then, we are interested in considering a model which has a similar structure of bivariate skew normal density of (2).

The purpose of this paper is to propose a new model which may be appropriate for a square ordinal table if it is reasonable to assume an underlying bivariate skew normal distribution with equal variances. Section 2 describes the new model and goodness-of-fit test, Section 3 shows some numerical simulations, and Section 4 analyzes the decayed teeth data using the proposed model.

2. New model and test

We consider a new model based on the skew normal distribution for square contingency tables with ordinal categories. In Section 2.1, we propose a skew normal distribution type symmetry model, namely SNDS. In Section 2.2, we describe a goodness-of-fit test for the model SNDS.

2.1. Skew normal distribution type symmetry model

We propose a new model for square contingency tables with ordinal categories as follows:

$$p_{ij} = 2\xi \alpha_1^{(i-j)^2} \alpha_2^{i-j} \beta_1^{(i+j)^2} \beta_2^{i+j} \Phi(i\lambda_1 + j\lambda_2) \quad (i = 1, \ldots, r; j = 1, \ldots, r),$$

where $\Phi(\cdot)$ is the cumulative distribution function of the standard univariate normal distribution, and $\lambda_1, \lambda_2 \in R$. Then we shall refer to this model as the skew normal distribution type symmetry (SNDS) model. It is easily seen that the SNDS model is an extension of the NDS model.
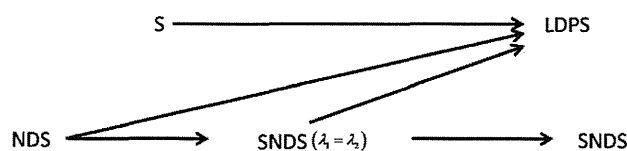
Fig. 1. Relationship among models. *Note:* $M_1 \rightarrow M_2$ indicates that model M_2 implies model M_1.

Let X and Y denote the row and column variables, respectively. Under the SNDS model, we see

$$\frac{p_{ij}}{p_{ji}} = \left(\alpha_2^2\right)^{i-j} \mu_{ij} \quad (i < j),$$

where

$$\mu_{ij} = \frac{\Phi(i\lambda_1 + j\lambda_2)}{\Phi(j\lambda_1 + i\lambda_2)}.$$

Note that only when $\lambda_1 = \lambda_2$, this structure is analogous to the LDPS structure. When $\lambda_1 = \lambda_2$, the SNDS model is a special case of the LDPS model. So, we also propose the SNDS model with $\lambda_1 = \lambda_2$ as the special SNDS model. For the special SNDS model, see Section 3. Under the SNDS model, if all $\{(\alpha_2^2)^{i-j}\mu_{ij} > 1\}$ hold, then $\Pr(X \leq i) > \Pr(Y \leq i)$, $i = 1, \dots, r-1$, hold. In addition, (1) if $\alpha_1, \beta_1, \beta_2 > 1, \alpha_2 < 1$ and $\lambda_2 > 0$, p_{ij} increases as the column value j increases, when the row value i is fixed, and (2) if $\alpha_1, \alpha_2, \beta_1, \beta_2 > 1$ and $\lambda_1 > 0$, p_{ij} increases as the row value i increases, when the column value j is fixed.

In addition, since the SNDS model is expressed by multiplying the form of the NDS model by cumulative distribution function of standard normal $\Phi(i\lambda_1 + j\lambda_2)$, we can consider the generalization of the SNDS model as follows:

$$p_{ij} = 2\xi\alpha_1^{(i-j)^2} \alpha_2^{i-j} \beta_1^{(i+j)^2} \beta_2^{i+j} F(i\lambda_1 + j\lambda_2) \quad (i = 1, \dots, r; j = 1, \dots, r),$$

where $F(\cdot)$ is the strictly increasing function with $0 < F(\cdot) < 1$. We shall refer to this model as the generalized NDS (GNDS) model. For the details of discussions using GNDS model, see Section 3.

Fig. 1 shows the relationship among the models considered here.

2.2. Goodness-of-fit test

We describe a goodness-of-fit test for a proposed model, named SNDS, in the previous section. Let n_{ij} denote the observed frequency in the (i, j)th cell of the table $(i = 1, \dots, r; j = 1, \dots, r)$ and let m_{ij} denote the corresponding expected frequency. Assuming that $\{n_{ij}\}$ have a multinomial distribution, the maximum likelihood estimates (MLEs) of expected frequencies $\{m_{ij}\}$ under the SNDS model could be obtained by maximizing the kernel of the log-likelihood function, subject to the constraint $\sum\sum p_{ij} = 1$, using the method of Lagrange multiplier. Namely, we must maximize the following function with respect to $\{p_{ij}\}$, λ and $\{\phi_{ij}\}$:

$$\sum_{i=1}^{r}\sum_{j=1}^{r} n_{ij} \log p_{ij} - \lambda\left(\sum_{i=1}^{r}\sum_{j=1}^{r} p_{ij} - 1\right) - \sum_{i=1}^{r}\sum_{j=1}^{r} \phi_{ij}\left(p_{ij} - 2\xi\alpha_1^{(i-j)^2} \alpha_2^{i-j} \beta_1^{(i+j)^2} \beta_2^{i+j} \Phi(i\lambda_1 + j\lambda_2)\right).$$

Then, using the Newton–Raphson method, we can obtain the MLEs of $\{m_{ij}\}$ and parameters $\xi, \alpha_1, \alpha_2, \beta_1, \beta_2, \lambda_1, \lambda_2$ under the SNDS model.

The likelihood ratio chi-squared statistic for testing goodness-of-fit of a model symbolized by M is

$$G^2 = 2\sum_{i=1}^{r}\sum_{j=1}^{r} n_{ij} \log\left(\frac{n_{ij}}{\hat{m}_{ij}}\right),$$

where $\hat{m}_{ij}$ is the MLE of m_{ij} under the model M. The numbers of degrees of freedom (df) for the LDPS, NDS and SNDS models are $(r - 2)(r + 1)/2$, $r^2 - 5$, and $r^2 - 7$, respectively.

3. Simulation studies

We present our investigation of the behavior of the new model for a bivariate skew normal distribution. Let the random variable $Z = (Z_1, Z_2)^T$ be distributed as a bivariate skew normal distribution with the density like Eq. (2). Suppose that there is an underlying bivariate skew normal distribution with some conditions and suppose that a 4×4 table is formed using cutpoints for each variable at -0.6, 0 and 0.6. Then, in terms of simulation studies, each subtable of Table 1 gives a 4×4 table of sample size 10 000, formed from an underlying bivariate skew normal distribution with various location, scale and skew parameters. Especially, in Table 1(a) and (b), there is an underlying bivariate skew normal distribution with skew parameters γ_1 and γ_2 being zero; i.e., there is an underlying bivariate normal distribution.

We see from Table 2(a) that both the NDS and SNDS models fit the data in Table 1(a) and (b) well. This seems natural because there is an underlying bivariate normal distribution in Table 1(a) and (b).

Table 1

The 4×4 tables of sample size 10 000, formed by using cutpoints for each variable at -0.6, 0 and 0.6 from an underlying bivariate skew normal distribution with various conditions.

(a) $\mu_1 = \mu_2 = 0$, $\sigma^2 = 1$, $\rho = 0.3$, $\gamma_1 = \gamma_2 = 0$				(b) $\mu_1 = 0.1$, $\mu_2 = 0.2$, $\sigma^2 = 1.5$, $\rho = 0.5$, $\gamma_1 = \gamma_2 = 0$			
1137	685	536	390	1090	597	510	683
704	559	512	514	500	329	341	589
540	504	519	679	446	346	397	758
432	491	696	1102	478	494	691	1751
(c) $\mu_1 = \mu_2 = 0$, $\sigma^2 = 1$, $\rho = 0.3$, $\gamma_1 = \gamma_2 = 0.3$				(d) $\mu_1 = \mu_2 = 0$, $\sigma^2 = 3$, $\rho = 0.3$, $\gamma_1 = \gamma_2 = 0.5$			
486	449	404	431	473	251	344	1165
432	447	481	678	290	155	177	670
415	520	599	919	353	182	217	805
419	660	961	1699	1155	651	735	2377
(e) $\mu_1 = \mu_2 = 0.1$, $\sigma^2 = 2$, $\rho = 0.3$, $\gamma_1 = \gamma_2 = 0.3$				(f) $\mu_1 = \mu_2 = -0.1$, $\sigma^2 = 2$, $\rho = 0.3$, $\gamma_1 = \gamma_2 = 0.3$			
632	348	410	900	758	487	476	930
329	205	251	694	446	264	270	569
386	254	295	791	435	290	292	706
864	633	767	2241	998	626	686	1767
(g) $\mu_1 = 0$, $\mu_2 = 0.2$, $\sigma^2 = 2$, $\rho = 0.3$, $\gamma_1 = \gamma_2 = 0.3$				(h) $\mu_1 = \mu_2 = 0$, $\sigma^2 = 2$, $\rho = 0.3$, $\gamma_1 = 0.1$, $\gamma_2 = 0.3$			
560	390	448	1092	836	484	558	1092
317	209	254	701	415	262	274	662
324	259	316	817	400	270	289	680
755	560	709	2289	785	549	659	1785
(i) $\mu_1 = 0.1$, $\mu_2 = 0.2$, $\sigma^2 = 2$, $\rho = 0.3$, $\gamma_1 = 0.1$, $\gamma_2 = 0.3$				(j) $\mu_1 = 0.1$, $\mu_2 = 0.2$, $\sigma^2 = 2$, $\rho = 0.3$, $\gamma_1 = 0.3$, $\gamma_2 = -0.3$			
676	437	527	1136	1137	403	344	559
343	225	259	699	575	278	242	438
359	240	293	779	576	293	283	528
677	531	687	2132	1213	733	740	1658

Table 2

The values of likelihood ratio chi-squared statistic G^2 for (a) the SNDS and NDS models, and (b) the GNDS model with some cumulative distribution functions, applied to the data in Table 1.

(a) G^2 for the SNDS and NDS models

Tables	G^2(SNDS)	G^2(NDS)
Table 1(a)	8.28	9.02
Table 1(b)	12.53	13.53
Table 1(c)	15.73	20.22*
Table 1(d)	12.93	20.73*
Table 1(e)	13.06	19.79*
Table 1(f)	13.15	22.59*
Table 1(g)	14.35	20.01*
Table 1(h)	12.90	20.50*
Table 1(i)	12.85	20.35*
Table 1(j)	16.11	20.03*

(b) G^2 for the GNDS model with the logistic, Cauchy and exponential distribution function

Tables	G^2(GNDS) (logistic)	G^2(GNDS)(Cauchy)	G^2(GNDS)(exponential)
Table 1(a)	8.36	8.50	6.12
Table 1(b)	12.81	12.73	13.53
Table 1(c)	15.63	15.81	15.58
Table 1(d)	13.19	13.72	13.03
Table 1(e)	19.05*	13.51	19.05*
Table 1(f)	14.30	16.65	10.59
Table 1(g)	15.10	16.49	13.89
Table 1(h)	13.35	14.43	12.01
Table 1(i)	13.53	15.02	10.54
Table 1(j)	16.31	16.94*	18.86*

* Means significant at the 0.05 level.

On the other hand, in Table 1(c) to (j) there is an underlying bivariate skew normal distribution with skew parameters γ_1 and γ_2 being non-zero. Then, we see from Table 2(a) that the SNDS model fits all the data in from Table 1(c) to (j) well, but the NDS model fits them poorly. Therefore, the SNDS model is more appropriate for a square contingency table if it is reasonable to assume an underlying bivariate skew normal distribution with equal marginal variances.

Table 3
Decayed teeth data of 349 men aged 18–39, for patients visiting a dental clinic in Sapporo City, Japan, from 2001 to 2005 (Tomizawa et al., 2006). (The parenthesized values are MLEs of expected frequencies under the SNDS model.)

Lower (numbers of decayed teeth)	Upper (numbers of decayed teeth)			Total
	0–4 (1)	5–8 (2)	9+ (3)	
0–4 (1)	115 (110.23)	55 (60.89)	25 (23.97)	195
5–8 (2)	16 (21.23)	49 (45.84)	60 (57.74)	125
9+ (3)	1 (0.45)	7 (4.46)	21 (24.19)	29
Total	132	111	106	349

Table 4
The values of likelihood ratio chi-squared statistic G^2 for models applied to the data in Table 3.

Models	df	G^2
Symmetry	3	98.24*
LDPS	2	3.72
NDS	4	20.17*
Special SNDS	3	20.17*
SNDS	2	4.72

* Means significant at the 0.05 level.

Next, we shall apply the GNDS model with $F(\cdot)$ to Table 1. Then we set the standard logistic and standard Cauchy distributions as the symmetric distribution with respect to zero, and standard exponential distribution as not-symmetric distribution, for the cumulative function $F(\cdot)$. We see from Table 2(b) that the GNDS models with the standard logistic and standard Cauchy distribution functions fit all the subtables in Table 1 well, and the values of G^2(GNDS) are very close to the values G^2(SNDS). This is because standard normal distribution function is similar to the standard logistic and standard Cauchy distribution functions. For the GNDS model with standard exponential distribution, the values of G^2 for the GNDS model are not different from the G^2 for the SNDS model so much. The GNDS model is an extension of the NDS model, and so the values of G^2 for the GNDS model are less than those for the NDS model. In addition, the term $F(\cdot)$ in the GNDS model may not be so different from the term $\Phi(\cdot)$ in the SNDS model, although standard normal distribution function itself is not similar to standard exponential distribution at all. It is thought that this is why the result described above is caused.

4. Analysis of decayed teeth data

We illustrate the techniques by applying the model of Section 2 to a real data by Tomizawa et al. (2006). The data are constructed from the data of the decayed teeth of 349 men aged 18–39, for the patients visiting a dental clinic in Sapporo City, Japan, from 2001 to 2005. Table 3 is classified by the number of decayed teeth on the lower side of the mouth of a patient and those on the upper side.

Table 4 gives the values of G^2 for models applied to the data in Table 3. We see from Table 4 that the NDS model fits the data poorly, on the other hand, the LDPS model fits them well. Thus we can guess that there is not an underlying normal distribution in Table 3, because both the NDS and LDPS models fit them well if there is an underlying normal distribution in the table. By the way, the SNDS model fits these data well, although the special SNDS model fits them poorly. So we shall investigate the data in Table 3 in more details using the SNDS model.

Under the SNDS model, the values of MLEs of parameters are $\hat{\alpha}_1 = 0.98, \hat{\alpha}_2 = 4.54, \hat{\beta}_1 = 1.09, \hat{\beta}_2 = 0.69, \hat{\lambda}_1 = -1.62, \hat{\lambda}_2 = 0.89$. So, we see $\{(\hat{\alpha}_2^2)^{i-j} \hat{\mu}_{ij} > 1\}$; i.e., $\Pr(X \le i) > \Pr(Y \le i)$, $i = 1, \ldots, r - 1$. Therefore, the number of decayed teeth in the upper side of the mouth of a patient tends to be more than that in the lower side.

5. Discussions

The existing NDS model may be appropriate for a square ordinal table if it is reasonable to assume an underlying *bivariate normal distribution* with equal marginal variances. On the other hand, the proposed model, i.e., the SNDS model may be appropriate for a square ordinal table if it is reasonable to assume an underlying *bivariate skew normal distribution* with equal marginal variances. Then note that the SNDS model is an extension of the NDS model. Therefore, for analyzing square contingency table data, it may be useful to apply the SNDS model to these data if it is not reasonable to assume an underlying bivariate normal distribution. Moreover, it is meaningful to use the SNDS model even if it is reasonable to assume an underlying bivariate normal distribution, because the SNDS model implies the NDS model, which may be appropriate when assuming an underlying bivariate normal distribution.

Finally, we note that we cannot always consider that there is an underlying bivariate skew normal distribution even if the SNDS model fits data well (also see Section 3).