usefulness of this procedure for patients with CRBSI, future studies with a larger group of patients will therefore be necessary.

When the previous published procedure was performed, there was a risk of losing the vascular side of the FS route [8–13]. Therefore, it is recommended to place two or three supporting sutures on the FS hold the FS in place [8]. However, the sutured wall of the FS is sometimes torn off and the vascular side of FS may shrink even with the supporting sutures [8], although the FS is known to form 2–4 wk after the insertion of CVC [17–19] and the strength of the FS wall is sufficient for 3 mo after the CVC insertion [14]. In the current procedure, only the anterior wall of the FS was cut after placing the supporting sutures. The remaining posterior wall was cut and removed when the procedure was completed to simplify the FS route. There was no loss or shrinkage of the FS among the patients using the current procedure, and the procedure was performed safely and easily.

Based on this clinical experience, the exchange procedure using the FS was effective for preserving the venous routes to the central vein in the patients who required long-term treatment, including PN. Furthermore, the procedure might be useful even in patients with CRBSI. A larger group of patients with CRBSI will therefore be necessary to elucidate the effectiveness of this procedure, as the current study involved only a small patient group.

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ORIGINAL ARTICLE

Identification of *TCTE3* as a gene responsible for congenital diaphragmatic hernia using a high-resolution single-nucleotide polymorphism array

Risa Teshiba · Kouji Masumoto · Genshiro Esumi · Kouji Nagata · Yoshiaki Kinoshita · Tatsuro Tajiri · Tomoaki Taguchi · Ken Yamamoto

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Abstract
Purpose Congenital diaphragmatic hernia (CDH) is a birth defect of the diaphragm associated with pulmonary hypoplasia. Although genetic factors have been suggested to play a role, the etiology of CDH is still largely unknown. In this study, we analyzed copy number variants (CNVs) using a single-nucleotide polymorphism (SNP) array to examine whether microdeletions contribute to the pathogenesis of this disease.
Methods A total of 28 CDH patients, including 24 isolated and 4 non-isolated cases, were available. We performed CNV analysis using high-resolution SNP arrays (370K, 550K, 660K; Illumina Inc.) and CNstream software. Deletions in loci that have been suggested in previous studies to contain candidate genes affecting CDH were analyzed.
Results We detected 335, 6 and 133 deletions specific for patients in 14 (350K array), 3 (550K) and 11 (660K) cases, respectively. Among these deletions, no segments included the previously suggested candidate genes with the exception of an 18-kb deletion observed in the candidate locus 6q27 in two non-isolated patients. This deleted region contains exon 4 of the *t-complex-associated-testis-expressed 3* (*TCTE3*) gene.
Conclusion Because *TCTE3* encodes a putative light chain of the outer dynein arm of cilia and human diseases caused by ciliary dysfunction show various phenotypes including skeletal defect, *TCTE3* may be a genetic candidate influencing CDH.

Keywords Congenital diaphragmatic hernia · Copy number variation · Microdeletion · Single-nucleotide polymorphism array · TCTE3

Introduction

Congenital diaphragmatic hernia (CDH) is a relatively common birth defect characterized by an abnormality in the integrity of the diaphragm. In a meta-analysis, its prevalence was calculated to be approximately 1/4,000 births [1]. CDH cases can be divided into two phenotypic categories—isolated and non-isolated—based on the presence of additional non-hernia-related anomalies. The non-isolated type makes up 30–40% of CDH cases [1–3], and some patients with this type show anomalies that are strongly suggestive of a recognized genetics syndrome such as Fryns syndrome (MIM 229850). In addition to a genetic syndrome within CDH, more than 50 CDH multiplex families have been reported [2]. These observations have implicated genetic factors in the condition's etiology.

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R. Teshiba · K. Yamamoto (✉)
Division of Genome Analysis, Research Center for Genetic Information, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
e-mail: kyama@bioreg.kyushu-u.ac.jp

R. Teshiba · K. Masumoto · G. Esumi · K. Nagata · Y. Kinoshita · T. Tajiri · T. Taguchi
Department of Pediatric Surgery, Reproductive and Developmental Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

K. Masumoto
Division of Thoracic, Endocrine and Pediatric Surgery, Faculty of Medicine, Fukuoka University, Fukuoka, Japan

Chromosomal aberrations have been identified as an important etiological factor in non-isolated CDH [4]. However, the majority of the cases were analyzed by low-resolution approaches, such as a combination of G-banded chromosome analysis and FISH. In recent years, array-based techniques have been widely used to detect small chromosomal changes that cannot be detected by traditional karyotype analysis, and a role for rare copy number variants (CNVs) in the etiology of diseases has gained considerable attention [5]. In non-isolated CDH, array-based analysis has detected several microchromosomal changes and delimited critical regions [6–10]. Those hotspot regions are of great interest because they may harbor genes associated with the development of CDH. However, the exact etiology of most cases of CDH still remains unknown.

In the present study, we screened CNV regions in CDH patients using a high-resolution single-nucleotide polymorphism (SNP) array to analyze the association of CNVs in both hotspot loci and candidate genes involved in retinoid signal pathways [11–13] with the disease.

Materials and methods

Subjects

This study was approved by the Ethics Committee at Kyushu University, Fukuoka, Japan. All patients or their parents were fully informed and agreed to participate in the study. DNA samples were obtained from 28 CDH patients; 24 had isolated CDH and 4 had CDH with additional anomalies. Non-isolated CDH cases were complicated with horseshoe kidney in one case, tracheal stenosis and double-outlet right ventricle (DORV) in one case, facial anomalies (low-set ears, micrognothia, saddle-nose) and cryptorchism in one case and Fryns syndrome in other case. The sides of defect were left-sided in 26 patients, right-sided in 1 patient and bilateral in 1 patient (Table 1). Patients with chromosomal aberrations already detected by karyotype analyses were excluded.

Table 1 Patient characteristics

Characteristic	Number of cases
Isolated	24
Non-isolated	4
Horseshoe kidney	1
Tracheal stenosis and double-outlet right ventricle	1
Fryns syndrome	1
Facial anomalies (low-set ears, micrognothia, saddle-nose) and cryptorchism	1
Defect side	
Left	26
Right	1
Bilateral	1
Total number of cases	28

DNA isolation and CNV analysis

Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp DNA Blood Midi Kit (QIAGEN) and was adjusted to a final concentration of 50 ng/μl for array analyses. The Illumina Human CNV370K-Duo, 370K-Quad, 550K-Duo or 660K-Quad Array and the Illumina BeadStation 500G SNP genotyping system were employed to obtain signal intensities of probes according to the manufacturers' protocols. To evaluate experimental quality, SNP genotypes were determined using BeadStudio Genotyping Analysis Module 3.3.7 software. All samples showed call rates greater than 0.99 (the average was 0.997; see supplemental Table S1).

CNstream was used to estimate CNVs from X and Y channel intensities loaded from the BeadStudio Genotyping data [14]. Input files for CNstream consisted of 14 patients into the 370K array, 3 into the 550K array and 11 into the 660K array, and each file also included 100 samples from normal controls. The numbers of markers for all autosomes were 334,515 in the 370K array, 547,405 in the 550K array and 639,815 in the 660K array. Those SNP and CNV markers successfully genotyped in <95% of cases were discarded. There were thus 345,359 markers remaining in the 370K array, 561,123 markers remaining in the 550K array and 656,308 markers remaining in the 660K array. Markers of sex chromosome were excluded from this study owing to inaccuracy of the computed result. CNVs were calculated using the CNstream program under the following parameters: maximum segment length (= 100 kb), number of probes per segment (= 5), minimum number of probes in one segment that must exceed the threshold for identifying an amplification/deletion (= 3), deletion threshold (= 1.65) and amplification threshold (= 2.7). The results of computation included the segments of CNV (bp), the percentage of amplifications and deletions over the samples (%) and the copy number assigned to each sample. CNstream could also perform an association analysis using Fisher's exact test and provide odds ratio and *P* values via a chi-squared case/control association test. The result was filtered by the percentage of amplification/deletion in cases and controls, and we selected deleted segments observed in case but not control samples. Selected segments were compared with the following sequences: (1) the candidate genes within the regions of chromosomal aberrations reported in CDH, the genes associated with the retinoid signaling pathway, and the genes whose mutations were reported in CDH; (2) the causative

genes of known syndromic CDH; and (3) the loci in which chromosomal abnormities were frequently reported. Physical position was obtained from the UCSC genome browser (http://www.genome.ucsc.edu/cgi-bin/hgGateway: NCBI36/hg18). Subsequently, the segments overlapping with those genes and loci were selected, and the logR ratio and B allele frequency of those segments checked using the BeadStudio genome viewer to exclude experimental artifacts.

Results

The CNstream program calculated the estimated amplification and deletion regions in both patients and control subjects, and a total of 21,366 segments were obtained in the 370K array, 1,945 segments were obtained in the in 550K array and 6,377 segments were obtained in the in 660K array (including redundant regions, Table 2). We presumed that the deletions not observed in the general population were relevant to the development of CDH. Therefore, deletions obtained in patients but not controls were subjected to evaluation. The numbers of such deletion segments were 335 in the 370K array, 6 in the 550K array and 133 in the 660K array.

Table 2 Output result of CNstream

SNP-array version	370K	550K	660K
Case/control	14/100	3/100	11/100
Number of markers	334,515	547,405	639,815
Segment of Amp./Del.			
Total number of detected segments	21,366	1,945	6,377
Amp. Case(+)/Control(−)[a]	20,333	682	1,224
Del. Case(+)/Control(+)[b]	540	73	2,064
Del. Case(+)/Control(−)[c]	335	6	133
(Per case)	(24)	(2)	(12)

Amp. amplification, *Del.* deletion

[a] Segments of amplification in cases but not in controls

[b] Segments of deletion in cases and controls

[c] Segments of deletion in cases but not in controls

The results of the comparison between the locations of deletion segments and candidate genes are shown in Table 3. With the exception of *HLX* and *DISP1*, these genes are associated with the retinoid signaling pathway. One mutation in and two variants of the *FOG2* gene were reported in isolated CDH [15, 16]. *HLX* is a homeobox gene transcription factor, and four variants were reported in isolated CDH as well [17]. The mutation of *DISP1* was reported in a CDH patient with additional malformations, and this gene is known to interact with sonic hedgehog [11]. So far, sequencing analyses have found no mutations in *COUP-TFII* [7, 9]. Deletion segments did not overlap with these genes.

Then, we compared these segments with genes known to be causative of monogenic syndromes (Table 4). Patients with these syndromes, which include Cornelia de Lange syndrome and Denys–Drash syndrome, are known to exhibit a higher incidence of CDH than the general population. However, the deletion segments did not overlap with the positions of these genes.

Table 3 Candidate genes for CDH and numbers of patients with detected deletion segments

Candidate gene (OMIM)	Band	Mutation (human)[a]	Study author	Number of patient with deletion segment[b]		
				370K	550K	660K
HLX (142995)	1q41	CDH−	Slavotinek et al. [17]	0/14	0/3	0/11
DISP1 (607502)	1q41	CDH+	Pober et al. [11]	0/14	0/3	0/11
RBP1 (180260)	3q23	–		0/14	0/3	0/11
RBP2 (180280)	3q23	–		0/14	0/3	0/11
LRAT (604963)	4q32.1	–		0/14	0/3	0/11
ALDH8A1 (606467)	6q23.3	–		0/14	0/3	0/11
GATA4 (600576)	8p23.1	–		0/14	0/3	0/11
FOG2 (603693)	8q23.1	CDH−	Bleyl et al. [15] Ackerman et al. [16]	0/14	0/3	0/11
RBP5 (611866)	12p13.31	–		0/14	0/3	0/11
RAIG1 (604138)	12p13.1	–		0/14	0/3	0/11
CRABP1 (180230)	15q25.1	–		0/14	0/3	0/11
COUP-TFII (107773)	15q26.2	Not found	Slavotinek et al. [7] Scott et al. [9]	0/14	0/3	0/11

CDH− isolated CDH, *CDH+* non-isolated CDH

[a] Reported mutations in human

[b] Number of patients in whom deletions for each gene were calculated by the CNstream program

Table 4 Monogenic syndromes in which CDH occurs and chromosomal aberrations are detected

Syndrome	Band	Gene	OMIM	Number of deletions in patients[a]
Cornelia de Lange syndrome	5p13.2	*NIPBL*	608667	0/28
	10q25.2	*SMC3*	606062	0/28
Denys–Drash syndrome	11p13	*WT1*	607102	0/28
Donnai–Barrow syndrome	2q31.1	*LRP2*	600073	0/28
Matthew–Wood syndrome	15q24.1	*STRA6*	610745	0/28
Spondylocostal dysostosis	19q13.2	*DLL3*	602768	0/28
	15q26.1	*MESP2*	605195	0/28
	7p22.2	*LFNG*	602576	0/28
	17p13.1	*HES7*	608059	0/28

[a] Number of patients in whom deletions for each gene were calculated by the CNstream program

Subsequently, we compared the location of these segments with loci at which chromosomal abnormalities have been frequently reported in non-isolated CDH (Table 5) [6, 7, 9, 18–30]. Although several segments overlapped with these loci, most of the overlapping regions were considered to be experimental artifacts after examination of the logR ratio and B allele frequency using BeadStudio platform. However, one region on 6q27 between cnvi0107052 and rs7767980; Chr6:169,866,304–169,884,311 (NCBI36/hg18) was obtained as deleted segment in two non-isolated CDH patients. One of these patients was clinically diagnosed with Fryns syndrome, and the other exhibited a dysmorphic face. Both of these patients died from having severe hypoplastic lungs. No common CNV in this region was found in the Database of Genomic Variants (DGV) (http://www.projects.tcag.ca/variation/) [31]. This 18-kb deleted region includes exon 4 of the *t-complex-associated-testis-expressed 3* (*TCTE3*) gene.

Discussion

Although there is a great deal of evidence implicating a genetic etiology in CDH and several genes have been shown to cause abnormal diaphragm development in mice, few CDH-related mutations have been identified in humans. Identifying chromosomal abnormalities in patients with non-isolated CDH has helped to map the candidate regions that might harbor CDH-related genes. Recently, array-based techniques have been widely used to detect micro-chromosomal changes that were previously undetectable karyotypically and to delimit the candidate regions.

Table 5 Frequently reported loci in which CDH occurs and chromosomal aberrations are detected

Locus[a]	dup, del	Study author	Number of deletions in patients[b]		
			370K	550K	660K
1q41–q42.12	del	Slavotinek et al. [7]	0/14	0/3	0/11
		Kantarci et al. [6]			
		Youssoufian et al. [18]			
3q21–q23	del	Wolstenholme et al. [19]	0/14	0/3	0/11
4q31.1–q32.3	del	Young et al. [20]	0/14	0/3	0/11
	del, dup	Celle et al. [21]			
6pter–p25.1	del	Batanian et al. [22]	0/14	0/3	0/11
		Baruch et al. [23]			
6q23–qter	del	Krassikoff et al. [24]	0/14	0/3	2/11[a]
		Shen-Schwarz et al. [25]			
8p23.1	del	Shimokawa et al. [26]	0/14	0/3	0/11
		Slavotinek et al. [7, 27]			
8q22.1–q24.13	del	Temple et al. [28]	0/14	0/3	0/11
15q23–q24.3	del	Sharp et al. [29]	0/14	0/3	0/11
15q26.1–q26.2	del	Scott et al. [9]	0/14	0/3	0/11
		Slavotinek et al. [7, 27]			
		Klaassens et al. [30]			

del deletion, *dup* duplication

[a] Frequently reported locus

[b] Number of patients in whom deletions within each locus were calculated by the CNstream programs

In the present study, we performed high-resolution CNV analysis using SNP arrays and a CNstream program specifically designed for Illumina microarrays. We detected the deletion of an 18-kb segment on 6q27 in two non-isolated patients but not in 100 normal controls. This small genomic region is not listed on the DGV. This region contains the *TCTE3* gene, which belongs to the dynein light chain family. The mouse homolog *TCTE3* gene is located in the *T/t*-complex of mouse chromosome 17. The *T/t*-complex is a genomic variant in mouse populations, and the t haplotype contains recessive lethal mutations that affect embryonic development [32]. *TCTE3*(−/−) mice have been generated, but they showed no apparent anomalies except for reduced flagellar motility [33, 34]. Therefore, a direct link between *TCTE3* and CDH was not evident in the mouse. However, *TCTE3* encodes a putative light chain of the outer dynein arm of cilia. The fact that human diseases caused by ciliary dysfunction show various phenotypes, including skeletal defects [35], suggests that this gene could be a candidate genetic factor responsible for some cases of CDH.

The length of the detected region was only 18 kb, and it is difficult to detect such a short segment using low-resolution analyses. Analyses with 660K or higher resolution became available only a few years ago, and the difference in resolution may explain why the deletion was undetected in the 370K and 550K arrays.

Many reports suggest that the retinoid signaling pathway plays an important role in the development of CDH. Of the genes involved in this pathway, *COUP-TFII* has been considered a particularly strong candidate because this gene was within the minimal region delimited by Klassenes et al. [30]. Slavotinek et al. [7] and Scott et al. [9] have performed sequencing analysis of *COUP-TFII* in a large number of CDH patients, but no mutations in this gene were found. On the other hand, a mutation and variants of *FOG2* and *HLX* genes were reported in isolated CDH cases [15–17]. In the present study, microdeletions in the genomic regions containing these genes were not identified. It is possible that sequence changes, but not segmental deletions of these genes, are responsible for the development of CDH. Another possibility is that the sensitivity of the array used in this study was limited by array's probe density. Combinatory analyses using re-sequencing and higher resolution array technologies will be required to better understand the genetic architecture involved in the pathogenesis of CDH.

Conclusion

A focal deletion of an 18-kb genomic region containing a part of the *TCTE3* gene on 6q27 was identified in two non-isolated CDH patients. Because the *TCTE3* belongs to the dynein light chain family and human diseases caused by ciliary dysfunction show various phenotypes (including skeletal defects), this gene could be a candidate genetic factor responsible for some cases of CDH.

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Conflict of interest The authors have nothing to disclose.

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ORIGINAL ARTICLE

Effect of insulin-like growth factors on lung development in a nitrofen-induced CDH rat model

Genshiro Esumi · Kouji Masumoto · Risa Teshiba · Kouji Nagata · Yoshiaki Kinoshita · Haruyoshi Yamaza · Kazuaki Nonaka · Tomoaki Taguchi

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Abstract
Purpose Both the mortality and morbidity associated with congenital diaphragmatic hernia (CDH) are mainly caused by pulmonary hypoplasia and persistent pulmonary hypertension. A previous study revealed that insulin-like growth factors (IGFs) play important roles in fetal lung development. The aim of this study was to investigate the effect of IGF-1 and IGF-2 on tissue cultures of fetal hypoplastic lungs obtained from nitrofen-induced CDH model rats.
Methods Pregnant rats were exposed to nitrofen on day 9 of gestation (D9). Fetuses were harvested on D18 by caesarian section. Lung specimens of the CDH (+) fetus were divided into three groups; control, IGF-1, and IGF-2. The specimens from the control group were cultured in culture medium without IGFs. The IGF-1 group specimens were cultured with IGF-1 (500 ng/ml), and those in the IGF-2 group were cultured with IGF-2 (500 ng/ml). The mRNA expression of TTF-1, T1α and α-SMA were analyzed in each group using real-time RT-PCR after 24 and 48 h of incubation. Immunohistochemical staining of these markers was also assessed for each of the cultured specimens.
Results There was a significant increase in the expression of both TTF-1 and T1α mRNA in the IGF-2 group, in comparison to the control group after 48 h of culture. Immunohistochemical staining revealed that the cell morphology was changed from cuboidal to squamous type in the IGF-2 group.
Conclusions An increased mRNA expression of the markers related to type 1 and 2 alveolar epithelial cells, and morphological changes in the epithelial cells were observed in the IGF-2 group. The administration of IGF-2 to nitrofen-induced hypoplastic lungs might lead to alveolar maturation, which thus results in their improved development.

Keywords Insulin-like growth factor · Lung development · Pulmonary hypoplasia · Congenital diaphragmatic hernia · Nitrofen

G. Esumi (✉) · K. Masumoto · R. Teshiba · K. Nagata · Y. Kinoshita · T. Taguchi
Department of Pediatric Surgery, Reproductive and Developmental Medicine, Faculty of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan
e-mail: esumi@pedsurg.med.kyushu-u.ac.jp

H. Yamaza · K. Nonaka
Section of Pediatric Dentistry, Division of Oral Health, Growth and Development, Faculty of Dental Science, Kyushu University, Fukuoka, Japan

Introduction

Congenital diaphragmatic hernia (CDH) still remains a challenge for neonatal surgeons. According to reports about the current postnatal therapies, the overall survival rate is now over 70% at some institutions [1, 2]. Nevertheless, CDH patients with severe pulmonary hypoplasia still have high mortality and morbidity rates [3]. To improve the outcome in such CDH patients with severe pulmonary hypoplasia, fetal treatment in order to accelerate the maturation process of hypoplastic lungs, especially in the later stages of pregnancy, is thought to be essential.

Various growth factors are considered to play important roles during the process of pulmonary organogenesis [4]. Among such growth factors, the effect of insulin-like growth factors (IGFs) on organ development has been

indicated throughout the perinatal period [5–11]. In addition, IGFs are known to be endocrine hormones that affect somatic changes, and have also been shown to affect cell proliferation and differentiation in some tissues via either autocrine or paracrine mechanisms [6]. In our previous study, the administration of either IGF-1 or IGF-2 to mouse lung explants demonstrated an increased in the number of type 2 alveolar epithelial cells (AECs-2) [12]. The biological actions of IGFs are transduced through specific surface receptors, insulin-like growth factors I receptor (IGF-1R), insulin-like growth factors-2 receptor (IGF-2R) and the insulin receptor (IR) [13]. Recent studies have revealed that these three receptors are all down-regulated in the hypoplastic lungs of the nitrofen-induced CDH model rat [14, 15]. Therefore, the IGFs are thought to be involved in the proliferation and differentiation of alveolar cells in fetal rat lungs.

We designed this study to investigate the hypothesis that the administration of IGFs improves the proliferation and differentiation during the lung development of the nitrofen-induced CDH model in the later stages of gestation. We investigated the mRNA expression of the markers of type 1 alveolar epithelial cells (AECs-1) and AECs-2 by real-time reverse transcription polymerase chain reaction (RT-qPCR). In addition, the localization of these marker proteins was also examined by immunohistochemical staining.

Materials and methods

Experimental animals

Pregnant Sprague-Dawley rats were purchased from a commercial breeder (Japan SLC, Inc., Shizuoka, Japan). The day when the vaginal plug was confirmed was designated as day 0 of gestation (D0). On D9 (term = D22), 100 mg nitrofen (Wako Pure Chemical Industries, Ltd., Osaka, Japan), which was dissolved in 1 ml of olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan), was given intragastrically. Fetuses were harvested on D18 by caesarian section. Lung specimens were dissected out from the CDH (+) fetuses, and were incubated in various media using an organ culture system.

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Kyushu University (approval No. A21-115-0).

Total RNA extraction and real-time RT-PCR

Total RNA was extracted from each specimen using the TRIzol® Plus RNA Purification Kit (Invitrogen, CA, USA) and PureLink™ DNase Set (Invitrogen, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using the SuperScript™ III First-strand Super Mix (Invitrogen, CA, USA). All samples were subjected to PCR amplification and were normalized to β-actin. All PCR reactions were performed using the LightCycler® Fast Start DNA Master SYBR Green I kit (Roche Applied Science, Mannheim, Germany). The accession numbers, primer sequences and product sizes for thyroid transcription factor-1 (TTF-1), podoplanin (T1α), α-smooth muscle actin (α-SMA) and β-actin are all listed in Table 1.

Tissue culture

Left lungs were dissected from D18 CDH (+) fetuses and were sliced into several specimens. Each specimen was placed on a polyethylene terephthalate filter (Falcon 353093 cell-culture insert, 8 μm pore size, on 353502 companion plate: BD, NJ, USA) and then was assigned to either the control, IGF-1, or IGF-2 group. The control group ($n = 6$) was cultured in DMEM/F-12 medium (Invitrogen, CA, USA) containing penicillin–streptomycin (100 U/ml and 100 mg/ml: Invitrogen, CA, USA), and L-ascorbic acid (150 μg/ml: Nacalai Tesque, Inc., Kyoto,

Table 1 Real-time RT-PCR primers

Gene	Accession number	Sequence (5′–3′)	Product size
TTF-1			
Forward	NM_013093.1	AAATTTGGGGGTCTTTCTGG	128
Reverse		AGAGTGCATCCACAGGGAAG	
T1α			
Forward	NM_019358.1	AAGCCTCAGATGACTCAATGACC	164
Reverse		ACTTTCTGAGCATCTGGGATGAG	
α-SMA			
Forward	NM_031004.2	GCTTCCTCTTCTTCCCTGGAG	105
Reverse		AGATGGCTGGAAGAGGGTCTC	
β-Actin			
Forward	NM_031144.2	TTGCTGACAGGATGCAGAAG	108
Reverse		TAGAGCCACCAATCCACACA	

Japan). The IGF-I group ($n = 6$) was cultured in media supplemented with 500 ng/ml of recombinant human IGF-1 (291-G1, R&D Systems, Inc., MN, USA). The IGF-2 group ($n = 6$) was cultured in media with recombinant human IGF-2 (292-G2, R&D Systems, Inc., MN, USA). These cultures were incubated for 24 or 48 h. The mRNA expression of TTF-1, as a marker of AECs-2, T1α, as a marker of AECs-1, and α-SMA, as a marker of smooth muscle, were analyzed in each group and were compared.

Immunohistochemistry

The cultured specimens were fixed in 4% paraformaldehyde phosphate buffer solution for 24 h. Thereafter, they were embedded in paraffin. Paraffin sections (5 μm) were dehydrated in xylene, rehydrated through alcohol, and incubated in methanol with 3% H_2O_2 to block endogenous peroxidase. All specimens were pretreated in 10 mM citrate buffer (pH 6.0) for 15 min using a microwave. The sections were then incubated with 10% normal goat serum and incubated overnight at 4°C with the primary antibodies. The lung specimens were stained with mouse monoclonal antibody to TTF-1 (sc-56606: Santa Cruz Biotechnology, Inc., CA, USA: dilution 1:50) to detect the AECs-2. T1α (11-035, anti-rat podoplanin monoclonal antibody: AngioBio Co., CA, USA: dilution 1:100) was stained to clarify the expression patterns of the AECs-1. In addition, an α-SMA antibody (monoclonal anti-α-SMA (1A4) antibody, Sigma, MO, USA: dilution 1:400) was also used to determine the degree of vascular development in the fetal lung (Table 2). These sections were then washed in phosphate-buffered saline (PBS) and incubated with biotinylated goat anti-mouse immunoglobulin G antisera. Then samples were washed in PBS and incubated with peroxidase-conjugated streptavidin. Immunohistochemical signals were visualized using 3,3′-diaminobenzidine substrate. Slides were counterstained with hematoxylin and examined by standard microscopy.

Statistical analysis

The mRNA expression of TTF-1, T1α and α-SMA from each of the groups at each culture time was analyzed and compared using a Student's t test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Relative mRNA expression levels of TTF-1, T1α and α-SMA

There was a significant increase in the expression of both TTF-1 and T1α mRNA in the IGF-2 group at 48 h of culture compared to the controls ($P = 0.018$ and 0.016) (Fig. 1a, b). The mRNA expression of α-SMA also increased in the IGF-2 group, but it did not reach statistical significance, compared to the control ($P = 0.06$) (Fig. 1c). At 48 h of culture, a significant difference in TTF-1 and T1α mRNA expression levels was also observed between the IGF-1 and IGF-2 groups ($P = 0.018$ and 0.008). However, no significant difference was observed between the IGF-1 group and the controls at any of the time points examined (Fig. 1a–c).

Table 2 Antibodies for immunohisitochemical staining

Marker	Clone	Working dilutions	Antigen retrieval	Source
TTF-1	SPM150	1:50	Citrate	Santa Cruz (CA, USA)
T1α	(11-035)	1:100	Citrate	AngioBio (CA, USA)
α-SMA	1A4	1:400	Citrate	Sigma (MO, USA)

Immunohistochemical analysis of the lungs after 48 h of culture

The TTF-1 protein, as a marker of AECs-2, was localized in the nuclei of the main bronchial and respiratory epithelial cells at all time points (Fig. 2a–c). The cuboidal TTF-1 positive cells on the distal lung epithelia decreased in number in the IGF-2 group. There were morphological changes in the TTF-1 positive cells, from a cuboidal to flattened form, only in the IGF-2 group (Fig. 2c, arrow).

The T1α protein, as a marker of AECs-1, was located on the surface of the epithelia in distal lung buds (Fig. 2d–f). There were no significant differences in the distribution among these groups with regard to the T1α staining.

The α-SMA was mainly distributed in the muscle layers of both arteries and bronchi. The protein was also located in the mesenchymal cells of the alveolar septa (Fig. 2g–i). There was no apparent difference in the α-SMA distribution among the three groups.

Discussion

The high mortality rate of human severe CDH patients is caused by hypoplastic lungs and the associated persistent pulmonary hypertension [3]. In hypoplastic CDH lungs, the maturation of epithelial cells is delayed, which is thought to be due to arrested development during the canalicular or saccular stage of the lung development [16, 17]. A reduction in bronchial divisions occurs in the hypoplastic lungs, resulting in the inhibition of a secondary increase in respiratory bronchi and the number of alveoli [18]. The alveoli in the hypoplastic lung were shown to be an

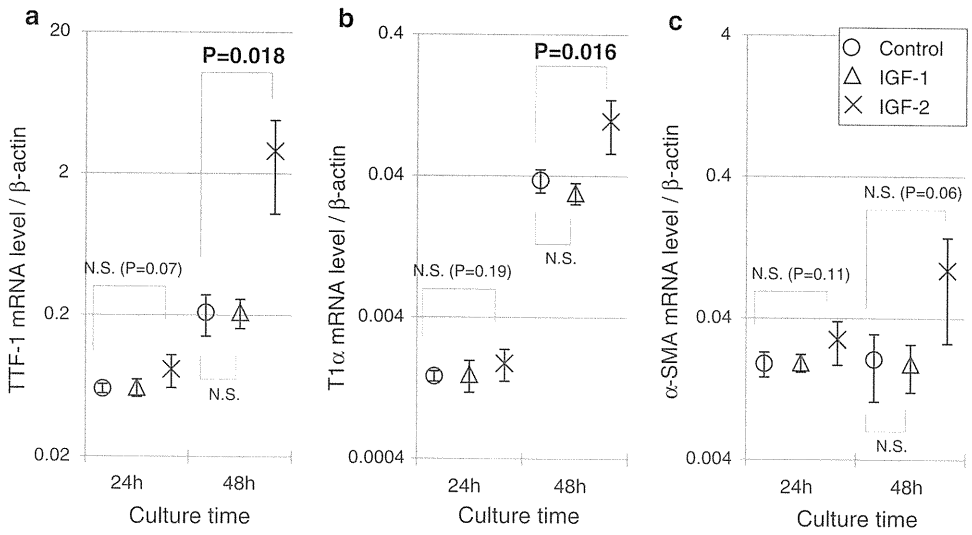

Fig. 1 Relative mRNA expression levels of TTF-1, T1α and α-SMA at 24 and 48 h of culture. The messenger RNA expression levels of TTF-1 (**a**), T1α (**b**), and α-SMA (**c**) are shown as averages (*open circle*, *open triangle*, *multi symbol*, respectively) and 95% confidence intervals (*bars*). Each chart has two time points (24, 48 h) and three groups (control, IGF-1, IGF-2). The expression levels of TTF-1 and T1α were significantly increased ($P = 0.018$ and 0.016) in the IGF-2 group at 48 h of culture, compared to the controls. The mRNA expression of α-SMA also was increased in the same group, although the increase did not reach statistical significance

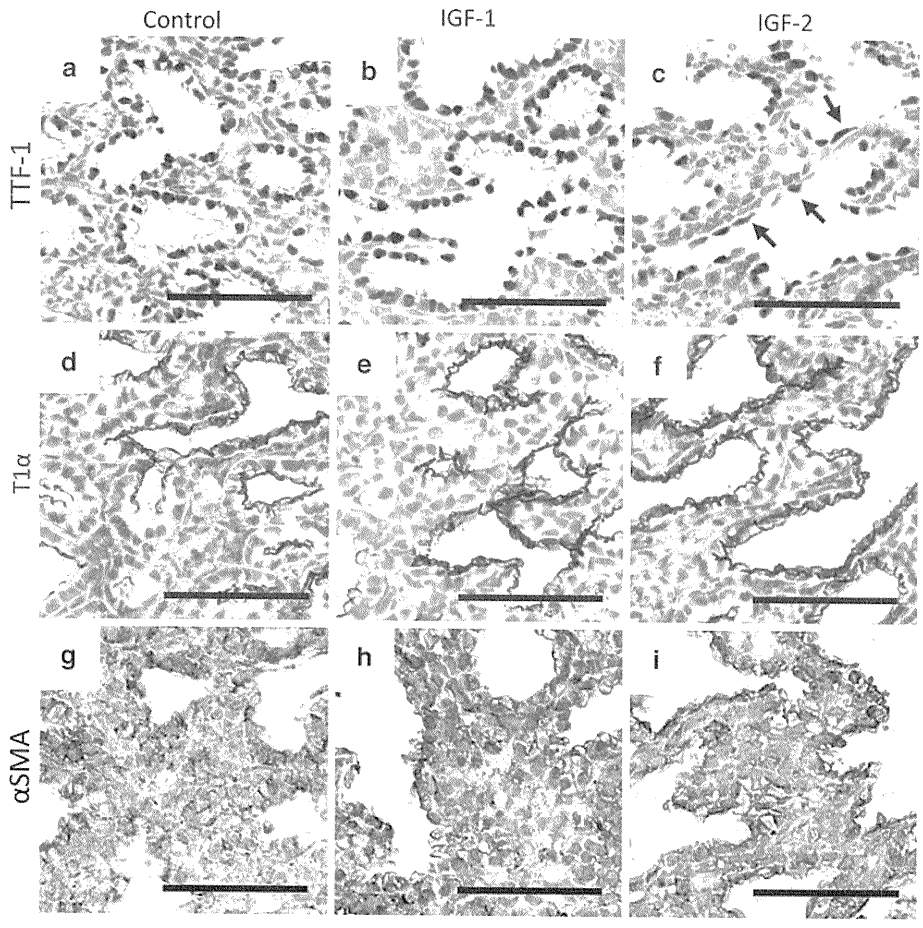

Fig. 2 An immunohistochemical analysis of the effect of IGFs at 48 h of culture. Immunohistochemical staining of TTF-1, T1α and αSMA was performed on the cultured specimens. In the TTF-1 staining (**a–c**), the number of cuboidal TTF-1 positive cells decreased in the IGF-2 group, and were replaced with the squamous type cells (**c**, *arrows*) after 48 h of culture. Such an effect was not observed in the other groups (**a**, **b**). There were no significant differences in either T1α (**d–f**) or α-SMA (**g–i**) staining among the groups. *Scale bars* 50 μm

immature form with thickened intraalveolar septa. The thickened intraalveolar septa cause a reduced capillary–air interface, which is essential for gas exchange [19]. Improvements in these factors are essential for improving the fetal outcome.

The differentiation from AECs-2 into AECs-1 is one of the key processes that occurs during the late gestational stage of lung development [20]. In addition, this differentiation has been reported to be impaired in the nitrofen-induced CDH model rat on D21 [21]. Retinoic acid has been demonstrated to be a promoter for this differentiation and also promotes pulmonary alveologenesis [22, 23]. In our previous study, the administration of either IGF-1 or IGF-2 to the cultured lungs of normal mouse fetuses at late gestation also demonstrated increased numbers of AECs-2 [12]. Therefore, we thought that the IGFs might induce the differentiation of alveolar epithelial cells during the late fetal period.

IGF-1 and IGF-2 are small peptide hormones which share 62% of their sequences, and they are homologous to proinsulin. IGF-1 and IGF-2, their receptors, and binding proteins, are all expressed in the fetal lungs of humans, rodents, and other species [8]. In addition, previous studies have shown that IGFs play important roles, especially in the late stage of lung development [7–10]. The deletion of either IGFs or IGFRs in transgenic mice causes the pulmonary hypoplasia or delayed lung maturation, respectively, thus resulting in fetal respiratory failure and perinatal death in both transgenic types [7–11]. In the targeted disruption of IGF-1, the phenotype of the mice included the following morphological findings: a lower airway volume, retarded epithelial growth, and a failure of capillary remodeling. Therefore, about 60% of IGF-1 knockout mice die because of respiratory failure during the perinatal period [7]. On the other hand, IGF-2 knockout mice demonstrate immature lungs with thicker alveolar septa and poorly organized alveoli, in comparison to the lungs of wild type mice [8]. Such mice, which had a null mutation of IGF-1R, tend to die just after birth due to respiratory failure [9]. In addition, IGF-2R knockout mice also die during the neonatal period due to the multiple organ malformations, including lung abnormalities [11]. These reports seem to support the hypothesis that addition of IGFs or an increase in the IGFs signaling in the lung might improve the outcome of CDH patients with severe pulmonary hypoplasia.

In this study, we investigated the effect of IGF-1 and IGF-2 on the fetal hypoplastic lungs taken from CDH model rat fetuses in the later stages of gestation using a tissue culture technique. Upon the administration of either IGF-1 or IGF-2 in the culture medium including the hypoplastic lung samples, only administration of IGF-2 demonstrated a significant increase in the mRNA expression of the markers related to both AECs-1 and AECs-2. Furthermore, in the immunohistochemical examination, the morphological changes of TTF-1 positive cells, from a cuboidal to squamous form, were also observed after 48 h of incubation with IGF-2. AECs-2 have been thought to be a progenitor of AECs-1, and the morphological changes of AECs are necessary for their maturation [20]. Therefore, the observed changes in cell form and the increase in the mRNA expression levels of markers of both AECs-1 and AECs-2 in the IGF-2 group are thought to reflect the maturation process of AECs. In contrast, IGF-1 did not demonstrate such an effect.

The biological functions of IGF-1 and IGF-2 are thought to be mediated through three receptors: IR, IGF-1R and IGF-2R. IGF-1R has been shown to have strong affinity for both IGF-1 and IGF-2. On the other hand, IGF-2R mainly binds IGF-2 with high affinity, while it interacts minimally with IGF-1 and insulin [24]. In the present study, only the IGF-2 group showed a positive effect on the rat CDH lung, suggesting that the effect was mediated by not only IGF-1R, but also IGF-2R. In fact, in the D18 CDH (+) rat lung, both IGF-1R and IGF-2R are shown to be distributed in both the alveolar epithelium and the mesenchymal cells [15]. Therefore, the administration of IGF-2 via amniotic fluid or an intratracheal space may be applicable to induce the improvement of lung maturation in the nitrofen-induced CDH model rat. In addition, IGF-1R and IGF-2R were both observed at term in normal rats (D21) [15]. Therefore, during the perinatal period, the rat lung might respond to the administration of IGFs.

In conclusion, our data showed that the administration of IGF-2 to nitrofen-induced hypoplastic lungs might lead to alveolar maturation in the later stages of gestation. Therefore, the administration of IGF-2 might result in improvements in lung development in premature fetuses in this stage. Based on our results, it is possible that fetal treatment with IGFs, especially IGF-2, may induce the lung maturation of hypoplastic CDH lungs. Therefore, perinatal treatment using IGF-2 might improve the outcome of severe CDH patients by improving the maturity of the hypoplastic lungs during the late fetal period.

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Short-term and long-term outcomes of 214 cases of non-immune hydrops fetalis ☆

Kotaro Fukushima [a,*], Seiichi Morokuma [a], Yasuyuki Fujita [a], Kiyomi Tsukimori [d], Shoji Satoh [e], Masayuki Ochiai [b], Toshiro Hara [b], Tomoaki Taguchi [c], Norio Wake [a]

[a] Department of Obstetrics and Gynecology, Kyushu University Hospital, Kyushu University, Fukuoka, Japan
[b] Department of Pediatrics, Kyushu University Hospital, Kyushu University, Fukuoka, Japan
[c] Department of Pediatric Surgery, Kyushu University Hospital, Kyushu University, Fukuoka, Japan
[d] Department of Obstetrics, Fukuoka Children's Hospital, Fukuoka, Japan
[e] Maternal and Perinatal Care Center, Oita Prefectural Hospital, Oita, Japan

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ABSTRACT

Despite advances in diagnosis and management, non-immune hydrops fetalis (NIHF) has a high mortality rate. Perinatal survival depends on the underlying disorder and the gestational age at diagnosis. As prognostic information is limited, this study acquired data regarding the neurological development of perinatal survivors. We performed a retrospective chart review of 214 cases in which NIHF was diagnosed antenatally. We recorded maternal demographic characteristics and interventions and their effectiveness, as well as the short-term outcome (survival) and long-term outcome including developmental quotients. Among the affected fetuses, 91 (42.5%) survived the perinatal period. Fetuses with chylothorax, chyloascites, or meconium peritonitis, and those in whom therapy was effective, had high survival rates irrespective of the type of intrauterine intervention. The subsequent intact survival rate was 28/56 (50.0%), with intact defined as ratio of the number of infants with normal development to the number of all infants followed. In contrast to the perinatal survival rate, the intact survival rate decreased as gestational age at diagnosis advanced. These findings suggest that the long-term intact survival rate depends on the underlying cause of NIHF. Additionally, while survival was improved with intensive perinatal care during the perinatal period, aggressive perinatal intervention was not a prognostic factor for neurological outcome.

1. Introduction

Hydrops fetalis is defined as abnormal fluid retention secondary to any pathophysiologic processes that occur during gestation. These etiologies are classified as immune or non-immune (NIHF) [1]. As hydrops secondary to Rh isoimmunization has become uncommon due to early diagnosis [5–7], most cases are now due to non-immune causes. Despite the introduction of several antenatal therapeutic interventions [1–4], however, the mortality rate of NIHF remains high.

The short-term prognosis of non-immune hydrops fetalis (NIHF) depends on the underlying cause and the gestational age at diagnosis [8–10]. There is, however, limited information regarding long-term prognosis and neurological development in perinatal survivors of NIHF.

In our retrospective chart review, we evaluated the effect of intrauterine therapy on short-term outcome (*e.g.*, survival) and long-term neurological development in infant survivors of NIHF managed in a Japanese tertiary care center.

2. Patients and methods

Women were followed at or referred to the Comprehensive Maternity and Perinatal Care Unit and Department of Obstetrics and Gynecology, Kyushu University Hospital, between January 1983 and March 2010. All patients were Japanese with the gestational age confirmed by multiple, sonographic fetal measurements during the first trimester. In 240 cases of antenatally diagnosed NIHF, 26 patients terminated the pregnancy, resulting in 214 cases available for analysis. This study was approved by the institutional review board.

A retrospective chart review recorded maternal demographic characteristics (*e.g.*, age, parity), antenatal intervention(s) and effectiveness, short-term outcome (*e.g.*, perinatal survival), and long-term outcome including developmental quotients (DQs) evaluated by the Enjoji Developmental Scale in Japanese [11,12]. Developmental delay or mental retardation was diagnosed when the DQ was less than 85 and 60, respectively.

The diagnostic and management protocol is summarized below. NIHF was defined as the presence of fetal subcutaneous tissue edema associated with a significant effusion in one or more cavities including the abdominal cavity and pleural or pericardial space, in the absence of atypical red cell antibodies. All fetuses underwent detailed structural and cardiac ultrasound examinations. Treponemal screening and rubella

☆ Sources of work: Kyushu University Hospital.
* Corresponding author at: Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 5395; fax: +81 92 642 5414.
E-mail address: kfuku@gynob.med.kyushu-u.ac.jp (K. Fukushima).

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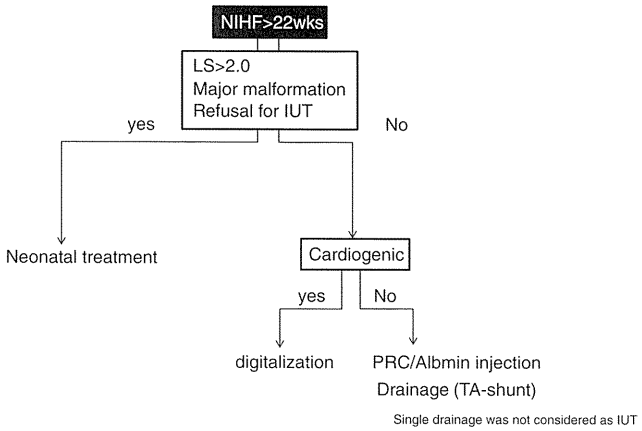


Fig. 1. Protocol for intrauterine intervention for fetuses with NIHF diagnosed after 22 weeks. Abbreviations: LS, lecithin/sphingomyelin ratio; IUT, intrauterine therapy; RC, red blood cell; TA, thoraco-amniotic shunt.

serology were performed on entry to prenatal care. Thoraco-abdominal centesis was utilized to diagnose chylothorax or ascites. Maternal serological testing for toxoplasmosis, cytomegalovirus, and parvovirus B19 as well as an amniocentesis for chromosomal analysis were also performed. The mid-cerebral artery peak systolic velocity (MCA-PSV) was also measured to diagnose anemia. Cordocentesis was performed in several cases that required transfusion. In this study, idiopathic hydrops was diagnosed as hydrops fetalis, in the absence of malformations, abnormal karyotype, chylothorax, or ascites.

A therapeutic protocol for the management of NIHF was designed under the assumption that three major etiologies exist for NIHF: fluid accumulation due to cardiac dysfunction, increased permeability due to decreased serum oncotic pressure, and exudation of lymphatic fluid (Fig. 1). Fetal cardiac dysfunction was defined as decreased cardiac function based on several ultrasonographic parameters in either ventricle coupled with a decreased amniotic fluid index. These cases were managed with transplacental digitalization and fetal albumin/red cell transfusions via the fetal abdomen or umbilical cord. For cases with non-cardiogenic NIHF, the pleural effusions were drained to increase osmotic pressure and prevent lung hypoplasia. Since the abnormal fluid collections were therapeutically aspirated at time of diagnostic sampling, we did not consider an isolated aspiration to be a therapeutic intervention. Antenatal intervention was considered effective if the extravascular fluid collections decreased in size, fetal cardiac function improved, or the MCA-PSV normalized (Fig. 1).

3. Results

The NIHF was diagnosed antenatally in all 214 cases by ultrasound scanning. The means for maternal age, parity, and the gestational age at the diagnosis of hydrops were 30.5 (range, 17–45) years of age, 1 (0–5), and 25.8 (12–39) weeks, respectively.

Of the total of 214 fetuses with NIHF, 91 (42.5%) survived the perinatal period. The remaining 123 fetuses died *in utero* or had perinatal deaths, with 35 (28.4%), 34 (27.1%) and 54 (43.9%) deaths *in utero* before 22 weeks of gestational age, after 22 weeks, or during the neonatal period, respectively. Of the 91 infants who survived the perinatal period, 79 (86.8%) were alive at one year (Fig. 2).

The main underlying disorders associated with intrauterine deaths at less than 22 weeks were cystic hygroma (21/36, 58.3%) and idiopathic (9/36, 25.0%). In contrast, the three major causes of death after 22 weeks were idiopathic (14/33, 42.4%), cystic hygroma (9/33, 27.3%), and cardiogenic (7/33, 21.2%) (Fig. 2, Table 1).

Short-term outcome was evaluated based on the gestational age at diagnosis (Table 2). Among the 62 cases detected before 22 weeks there were 12 perinatal survivors (18.8%). Among 30 cases detected between 22 and 25 weeks there were 10 perinatal survivors (33.3%). The perinatal survival rate among the 56 cases detected between 26 and 29 weeks (25/56, 44.6%). Finally, among the 66 cases detected after 30 weeks there were 44 survivors for a perinatal survival rate of 66.7%.

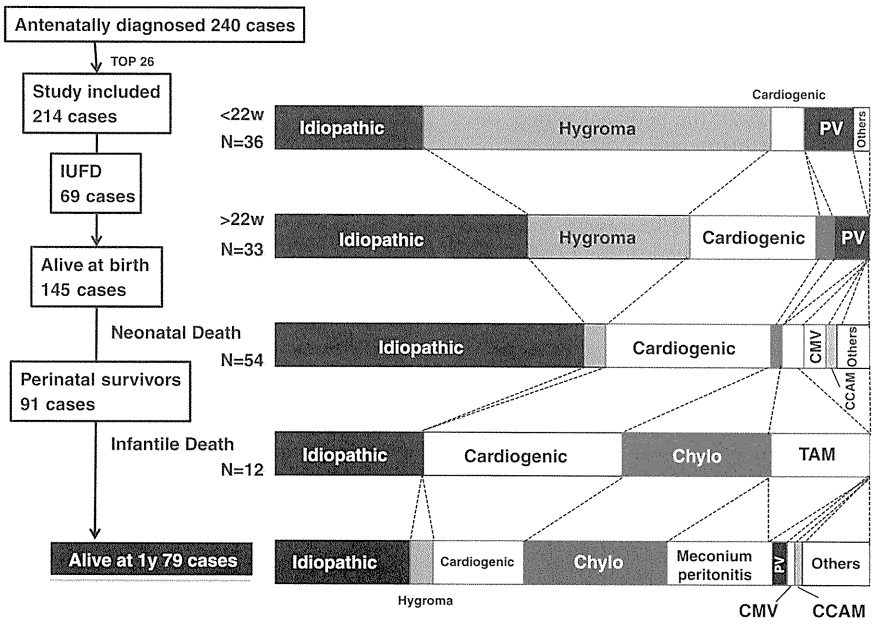


Fig. 2. Underlying disease and short-term outcome for fetuses with non-immune hydrops fetalis (NIHF). The bar graph shows the ratio of different underlying diseases in patients with an intrauterine fetal death before 22 weeks, after 22 weeks, perinatal death, death in infancy, and in survivors alive beyond one year. Abbreviations: TOP, termination of pregnancy; PV, parvovirus-induced anemia; chylothorax or chyloascites; TAM, transient abnormal myelopoiesis; CMV, cytomegalovirus infection; CCAM, congenital cystic adenomatoid malformation of lung; IUFD, intrauterine fetal death.

Table 1
Outcome and background disorder.

Disorder	Total		Number of aneuploidy (21 trisomy)	Ratio of aneuploidy (examined cases)	IUFD		Neonatal death	Perinatal survivors		Perinatal survival rate (%)	Normal development (followed up patient)	Remarks
					<22	>22		Infantile death	Alive at 1 year			
Idiopathic (unknown)	72	33.6%	25 (23)	44.6% (56)	9	14	28	3	18	29.2	45.5%, 5(11)	
Cardiogenic	40	18.7%	6 (5)	24.0% (25)	2	7	15	4	12	40.0	37.5%, 3(8)	
Hygroma	35	16.4%	9 (3)	60.0% (15)	21	9	2		3	8.6	50%, 1(2)	Exclude 24 TOPs
Chylothorax chyloascites	24	11.2%	7 (3)	35.0% (20)		1	1	3	19	91.7	50%, 8(16)	
Meconium peritonitis	14	6.5%	0	0% (7)					14	100.0	88.9%, 8(9)	
Parvovirus-induced Anemia	7	3.3%	0	0% (7)	3	2			2	28.6	100%, 2(2)	Exclude 1 TOP
TAM	4	1.9%	4 (4)	100% (4)			2	2		50.0	–	
CCAM	3	1.4%	0	0% (1)			2		1	33.3	100%, 1(1)	Exclude 1 TOP
CMV	2	0.9%	0	0% (2)			1		1	50.0	0	
Others	13	6.1%	2 (0)	28.6% (7)	1		3		9	69.2	14.3%, 1(7)	
Overall	214		53 (38)	36.8% (144)	36	33	54	12	79	42.5	50.0%, 28(56)	

TAM transient abnormal myelopoiesis, CCAM: congenital cystic adenomatoid malformation of lung, CMV: cytomegalovirus infection, TOP: termination of pregnancy, IUFD: intrauterine fetal death.

Table 2
Outcome and gestational week at diagnosis.

Gestational week at diagnosis	Total		IUFD (<22w)	Neonatal death	Neonatal death		Perinatal survival rate (%)	Normal development (followed up patient)	Remarks
					Infantile death	Alive at 1 year			
<22	62	29.0%	49 (35)	1	1	11	18.8	71.4%, 5 (7)	Exclude 26 TOPs
22–25	30	14.0%	11	9	–	10	33.3	60.0%, 3 (5)	–
26–29	56	26.2%	7	24	2	23	44.6	50.0%, 7 (14)	–
30–	66	30.8%	2	20	9	35	66.7	43.3%, 13 (30)	–
Overall	214		69	54	12	79	42.5	50.0%, 28 (56)	–

TOP: termination of pregnancy, IUFD: intrauterine fetal death.

As shown in Table 3, the distribution of the gestational age at diagnosis differed by underlying disease. The median age (range in weeks) for idiopathic, cardiogenic, cystic hygroma, chylothorax/ascites, meconium peritonitis, parvovirus-induced anemia, transient abnormal myelopoiesis (TAM), congenital cystic adenomatoid malformation of lung (CCAM), and cytomegalovirus were 26 (14–37), 28 (14–38), 17 (12–31), 31(20–37), 28 (23–39), 25 (19–31), 31 (29–33), 27 (26–30) and 22 (19–25), respectively.

Among the 91 fetuses who survived the perinatal period, cases with chylothorax, chyloascites, and meconium peritonitis had high survival rates irrespective of intrauterine therapy (Table 4, Fig. 2). Digitalization for cardiogenic NIHF had high survival ratios when therapy was effective, with rates of 100% (5/5 cases). A thoracoamniotic shunt was placed in three cases (two cases of chylothorax and one case of CCAM) and was effective in all cases. A spontaneous decrease in fluid was seen in 33.3% (7/21) of the perinatal survivors with idiopathic NIHF.

To date, 56 (including 2 cases with 45 XO and 4 cases of 21 trisomy) of 79 infant survivors have been followed for more than one year (Table 5). The remaining cases were excluded from long-term outcome analysis due to age below one year or discontinuation of follow-up. In the 58 analyzed cases, 19 infants showed neurologic impairment based on DQ (32.8%). Nine patients (15.5%) had suspected cerebral palsy and/or suspected mental retardation or showed subnormal development. Thus, the subsequent intact survival rate for the survivors of NIHF was 30/58 (51.7%).

The breakdown for intact survival (the ratio of survivors with normal development to the total number of followed survivors) by underlying disease is shown in Table 1. There were no significant differences in terms of intact survival for any diseases except for meconium peritonitis.

In contrast to the finding on gestational age at diagnosis and perinatal survival rate, we found that the ratio of intact patients decreased as the gestational age at diagnosis advanced. Intact survival rates in cases detected before 22 weeks, between 22 and 25 weeks, between 26 and 29 weeks, and after 30 weeks were 71.4%, 60.0%, 50.0%, and 43.3%, respectively.

Although numerous underlying disorders were associated with non-intact survival, other factors related to neurological outcome, including low birth weight, chromosomal aberration or traumatic birth injury were identified in 11 of 19 cases (57.9%) (underlined in Table 5). When stratified for the use of intrauterine therapy, four of nine of the survivors who responded to therapy demonstrated neurological impairment.

Table 3
Relationship between background disease and gestational age when diagnosed as hydrops.

Disorder	Total	<22	22–25	26–29	30–	Median, (range)
Idiopathic (unknown)	72	19	14	19	20	26, (14–37)
Cardiogenic	40	6	3	17	14	28, (14–38)
Hygroma	35[a]	29	4	1	1	17, (12–31)
Chylothorax chyloascites	24	1	1	5	17	31, (20–37)
Meconium peritonitis	14		3	5	6	28, (23–39)
Parvovirus-induced anemia	7[b]	5	1	1		20, (14–28)
TAM	4			1	3	31, (29–33)
CCAM	3[b]			2	1	27, (26–30)
CMV	2	1	1			22, (19–25)
Others	13					27, (17–33)
Overall	214	62	30	56	66	

TAM transient abnormal myelopoiesis, CCAM: congenital cystic adenomatoid malformation of lung, CMV: cytomegalovirus infection, TOP: termination of pregnancy, IUFD: intrauterine fetal death.
[a] Exclude 24 TOPs.
[b] Exclude 1 TOP.

Table 4
Disorders and therapeutic intervention in perinatal survivors.

	Perinatal survivors	Intervention (effective)	albumin	BT	TA-Shunt	Drainage[a]	Digitalization	Spontaneous diminishment
Chylothorax/ascites	22	14 (2)	1		2 (2)	14		
Idiopathic/unknown	21	8 (2)				8 (2)		7
Cardiogenic	16	8 (5)				3	5 (5)	2
Meconium peritonitis	14	7	1			6		2
Others	7	0		2[b] (1)				
Hygroma	3	1				1		1
TAM	2	0						
Parvovirus-induced anemia	2	2		2 (2)				0
CMV	1	0						1
CCAM	1	1			1 (1)			

TAM transient abnormal myelopoiesis, CCAM: congenital cystic adenomatoid malformation of lung, CMV: cytomegalovirus infection, BT: intrauterine blood injection, TA: thoraco-amniotic. The intrauterine interventions were considered effective if fluid reduced or when fetal cardiac function improved.
[a] Both cases were diagnosed as congenital anemia.
[b] Single drainage was not considered as therapeutic intervention.

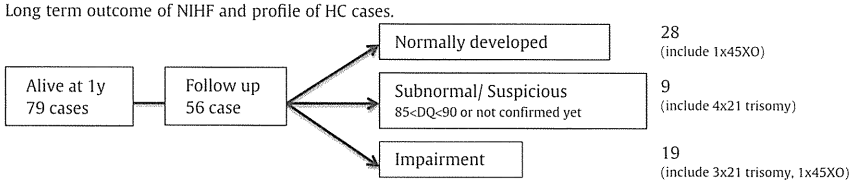
4. Discussion

Until a few decades ago, the majority of cases of hydrops fetalis were attributable to Rh isoimmunization. Now, almost all cases are non-immunologic in origin [5–7]. As NIHF includes a variety of etiologies; prognosis, outcomes, and therapeutic options range widely [13]. Since 1980, our institution has attempted to register cases of hydrops fetalis and offer *in utero* therapy. We now review the clinical characteristics and short- and long-term outcomes of cases of NIHF managed at our institution.

The common etiologies of NIHF include cardiogenic (congenital heart problems, arrhythmia), congenital anomalies, chromosomal abnormalities, congenital viral infections, congenital anemia, and congenital chylothorax or chyloascites [7]. Despite advances in diagnosis and neonatal management, the outcome in NIHF remains poor [14]. In our series, the perinatal survival rate was 41.4%.

Similar to previous studies [7], we noted that the prognosis of NIHF depended on the cause. Cystic hygroma was the main underlying disorder for intrauterine fetal deaths at less than 22 weeks, accounting for over two thirds of cases. Cystic hygroma is often associated with other major structural abnormalities and/or chromosomal aberrations. Termination of pregnancy is a consideration for these patients [15]. In contrast, the perinatal survival rate among fetuses with chylothorax/ascites or meconium peritonitis was much better (91.7% and 100%). Prognosis also depends on the gestational age at the onset of hydropic change, with earlier emergence being associated with a worse outcome [9]. This correlation seems to be due at least in part to an association between the specific background disease

Table 5
Long term outcome of NIHF and profile of HC cases.



Maternal age	Diagnosed (w)	Delivery (W)	Birth weight	Apgar (5)	UmpH	Diagnosis	IUT[a]	Effect	Effect follow up length	DQ	Neurological development
24	37	37	3112	8	7.297	Chylothorax	–		10	73	DD
29	35	36	3245	NA	7.336	Chylothorax	–		10	83	DD
31	29	29	1420	9	NA	CAVB	–		8	73	DD
26	38	38	2618	NA	7.375	Meconium peritonitis	–		7		CP
28	23	28	1826	5	7.201	Lymphangioma	–		5	62	DD
29	36	37	3150	NA	7.34 21	Trisomy	–		4	NA	MR
34	30	32	1330	3	7.263	Hemolytic anemia	–		4	40	CP + MR
23	27	38	3240	7	7.177	CDH, 21 trisomy	–		3	59	MR
40	29	33	1902	8	7.344	Chylothorax, 21 trisomy	TAS	Yes	2	NA	DD
37	30	36	3048	7	7.367	Pena schokeir syndrome	–		2	55	MR
23	23	33	1780	5	7.298	CAVB,ECD,PS,AS	Di	Yes	2	81	DD
27	28	32	2338	4	7.295	Parvovirus-induced anemia, traumatic delivery	BT	Yes	2	19	CP + MR
32	30	31	1390	8	7.225	TOF, ASD, multiple anomalies	–		2	21	CP + MR
26	29	33	2644	4	7.366	Cloaca anomaly	–		2	NA	CP + MR
34	31	33	2078	7	7.138	PVL	–		2		CP
25	29	34	2978	8	7.418	45XO, chylothorax, ASD	–		1	67	DD
29	38	38	3400	7	7.286	Ebstein's anomaly, TR, PS	–		1	69	DD
32	32	34	2410	2	7.364	Ventriculomegaly	–		1	4	CP + MR
33	26	34	3262	5	7.343	NLE, AS, PSVT, chylothorax	Di	Yes	1	84	DD

NA: not available, TAS: thoraco-amniotic shunt, Di: digitalization, BT: intrauterine blood injection, CAVB: congenital atrioventricular block, CDH: congenital diaphragmatic hernia, ECD: endocardial cushion defect, PS: pulmonary stenosis, TOF: Tetralogy of Fallot, AS aortic stenosis, ASD: atrial septal defect, PVL: periventricular leukomalacia, TR: tricuspid regurgitation, NLE: neonatal lupus erythematosus, PSVT: paroxysmal supraventricular tachycardia, CP cerebral palsy, MR: mental retardation (DQ<60), DD: developmental delay (DQ<60–85).
[a] Single drainage was not considered as IUT.

and the typical gestational age at diagnosis, as is the case with cystic hygroma.

Several *in utero* therapeutic options have been tried with varying success rates [13,16]. Effectiveness is lowest for idiopathic NIHF. Prognosis presumably will be improved if the pulmonary complications and cardiac failure, which are sequelae to the structural damage secondary to inappropriate fluid retention, are aggressively managed.

A previously published review of long-term outcomes in 19 children with NIHF who survived beyond one year of age showed that 13 (68.4%) children developed normally, two had mild developmental delay, and four were severely delayed [17]. Our intact survival rate (50.0%) was slightly lower; however, our considerably larger sample size makes a direct comparison of survival rates less valid. As with any retrospective study, a significant limitation is the quality of the medical record. Additionally, our results may have been affected by selection bias as patients diagnosed early in pregnancy may have chosen pregnancy termination.

Like the perinatal mortality rate, the long-term intact survival rate after NIHF also depended on the type of underlying disorder. For example, fetuses affected by parvovirus B19 had a high rate of intact survival consistent with what has been previously reported [18]. In contrast, fetuses with chylothorax/ascites did poorly. In addition, the non-intact survivors in our series commonly had risk factors including very low birth weight, chromosomal aberration, or possible hypoxic episodes *in utero*. Interestingly, in contrast to the perinatal survival rate, the intact survival rate decreases as the gestational age at which NIHF is diagnosed advances [18]. This finding, which we corroborated, suggests that the long-term intact survival rate after NIHF depends in part on the underlying etiology. Those fetuses with a mild or easily correctable underlying disorder may be rescued by intensive perinatal care. Nakayama et al. proposed that the prevention of premature labor and associated complications such as birth injury and chronic *in utero* hypoxia might reduce neurological impairment [19]. Poor condition at birth is also a strong predictor of death in patients with NIHF [14]. Although a more precise follow-up study is necessary, this finding might indicate that a good prognosis is likely for selected fetuses who receive effective intensive care during the perinatal period. A good neurological outcome does not, however, go hand-in-hand with a good perinatal prognosis as in the case of chylothorax diagnosed at 30 weeks of gestation.

While the underlying etiology clearly affects prognosis, caution must be used with respect to interpreting our results for each etiology individually as the numbers of cases in each category are small. In addition, only 79 out of the 214 (36.9%) were alive at one year and therefore our conclusions regarding survival beyond one year are based on a relatively small sample. Another limitation is that significant changes in neonatal management, indications for delivery, methods for fetal therapy, and diagnostic procedures have occurred in the last 27 years over which the cases were acquired. Despite these limitations, this report is one of the largest series investigating outcomes in NIHF and provides valuable information regarding the neurological prognosis in NIHF.

Advances in fetal medicine including intrauterine fetal transfusions for anemia or thoracocentesis for pleural effusions have improved the prognosis of NIHF. Patients should be counseled regarding prognosis in the context of the underlying etiology. Additionally, other factors such as karyotype should also be considered [10]. Our series included 25 cases with abnormal karyotypes in the idiopathic category; however, 23 of these 25 cases were patients with 21 trisomy without any other morphological abnormalities. Of those infants who survived over a year, however, 47 of 56 (83.9%) patients had a normal karyotype (see Table 5). Thus given the poor prognosis, patients presenting with a diagnosis of NIHF and an abnormal karyotype at an early gestational age will often elect termination.

The decision to deliver should involve a multidisciplinary approach with contributions from a maternal-fetal medicine specialist as well as a neonatologist. Finally, a planned delivery in a tertiary center will result in optimal resuscitation and neonatal care [10,20,21].

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Applied nutritional investigation

Successful treatment of an infected wound in infants by a combination of negative pressure wound therapy and arginine supplementation

Kouji Masumoto M.D., Ph.D. [a,b,*], Kouji Nagata M.D. [a], Yoichiro Oka M.D. [b], Hiroki Kai M.D., Ph.D. [b], Sadako Yamaguchi R.D. [c], Mika Wada R.N., WOON. [c], Tsuyoshi Kusuda M.D. [d], Toshiro Hara M.D., Ph.D. [d], Shin-ichi Hirose M.D., Ph.D. [e], Akinori Iwasaki M.D., Ph.D. [b], Tomoaki Taguchi M.D., Ph.D. [a]

[a] *Department of Pediatric Surgery, Reproductive and Developmental Medicine, Graduate School of Medical Sciences, Kyushu University, Kyushu, Japan*
[b] *Department of Thoracic, Endocrine and Pediatric Surgery, Faculty of Medicine, Fukuoka University, Fukuoka, Japan*
[c] *Nutrition Support Team, Kyushu University Hospital, Kyushu, Japan*
[d] *Neonatal Intensive Care Unit, Kyushu University Hospital, Kyushu, Japan*
[e] *Department of Pediatrics, Faculty of Medicine, Fukuoka University, Fukuoka, Japan*

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ABSTRACT

Objective: Wound dehiscence caused by surgical site infection (SSI) presents a complicated problem. Negative pressure wound therapy (NPWT) was developed to treat wound dehiscence. Nutritional treatment using arginine has also been recently shown to be effective for the treatment of pressure ulcers. Therefore, wound complications due to SSI were treated using NPWT combined with nutritional therapy with an arginine-rich supplement (ARS).
Methods: Six pediatric patients with wound dehiscence due to SSI received this combined therapy.
Results: The average age of the patients was 12.2 mo. The operations that these patients underwent included laryngotracheal separation, radical operation for spinal bifida, gastrostomy, colostomy, anorectoplasty, and tumor extirpation. A local wound infection induced wound dehiscence in all patients. Therefore, NPWT was introduced with an enteral administration of ARS. All wounds completely healed within 1 mo after the introduction of this combined therapy without any other complications from the NPWT or ARS. A follow-up study at 6 mo after this therapy was completed showed no complications associated with the wounds.
Conclusion: This combination therapy using NPWT and ARS administration was effective in inducing early healing of infected wound complications after surgery.

Introduction

Wound dehiscence caused by surgical site infection (SSI) presents a complicated problem for surgical clinicians, despite the improvements in surgical procedures and infection control techniques. The Center for Disease Control in the United States has reported that SSI is the third most frequently reported type of nosocomial infection, accounting for 14% to16% of such infections in hospitalized patients and 38% in surgical patients [1]. SSI is defined as an infection occurring within 30 d after a surgical operation (or within 1 y if an implant is left in place after the procedure) and affecting the incision or deep tissue at the operation site [2]. The infections are caused by multiple risk factors related to the patient, the procedure, and the hospital environment. The responsible pathogens in SSI can originate from the patient's endogenous flora or exogenous sources [2–4].

Negative pressure wound therapy (NPWT) was developed for wound dehiscence and impaired wound healing [5–12]. Nutritional treatment using arginine also has been found effective for the treatment of pressure ulcers and wound healing after traumatic hemorrhage [13–20]. Therefore, wound complications caused by SSI were managed using a combination therapy of NPWT and an arginine-rich supplement (ARS) in the present study.

Materials and methods

Patients

Six infant patients who had been treated from January 2008 through February 2010 in the Department of Pediatric Surgery of Kyushu University or

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* Corresponding author. Tel.: +81-92-801-1011; fax: +81-92-861-8271.
E-mail address: kmasu@fukuoka-u.ac.jp (K. Masumoto).

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Table 1
Background of patients with wound dehiscence

Patient number	Sex	Age	Baseline condition	Operation	Associated conditions
1	F	26 mo	laryngeal dysfunction	laryngotracheal separation	omphalocele, thoracic hypoplasia, pulmonary hypoplasia, chronologic anomaly
2	M	0 d	spinal bifida	radical operation	tethered cord syndrome
3	F	31 mo	dysphagia	gastrostomy	CHARGE association, tracheomalacia
4	M	6 mo	rectovesical fistula	reconstruction of colostomy	bilateral VUR and megaureter
5	M	10 mo	rectovesical fistula	anorectoplasty	bilateral VUR
6	F	0 d	sacrococcygeal teratoma	tumor extirpation	none

F, female; M, male; VUR, vesicoureteral reflux

the Department of Thoracic, Endocrine, and Pediatric Surgery of Fukuoka University and demonstrated wide wound dehiscence cause by SSI were enrolled in this study. The combination therapy using NPWT and ARS administration was started when the SSI was recognized and continued until the wound dehiscence was completely closed. Table 1 presents the basic characteristics of patients in this study. The baseline conditions were laryngeal dysfunction, spinal bifida, dysphagia due to multiple anomalies, sacrococcygeal teratoma, and two types of anorectal anomaly. The associated anomalies included omphalocele, thoracic hypoplasia, pulmonary hypoplasia, and a chronologic anomaly in one patient, tethered cord syndrome in one patient, CHARGE association and tracheomalacia in one patient, and bilateral vesicoureteral reflux in two patients. The age at time of surgery ranged from 1 d to 26 mo. The mean age at the time of surgery was 12.2 mo. The operations included laryngotracheal separation, radical operation for spinal bifida, gastrostomy, reconstruction of a colostomy, anorectoplasty for rectovesical fistulas, and tumor extirpation. There were different baseline conditions observed because the investigation was conducted over a short period and patients with the same condition could not be selected for this study.

All patients enrolled in this study were without systemic infections at the start of NPWT and ARS administration other than wide wound dehiscence caused by SSI. Patients with severe hepatic or renal dysfunction, a metabolic disorder of amino acids, or hypersensitivity or allergy to arginine were excluded from this study. All parents of the candidates were informed of the therapy before the study started and provided informed consent for their children to participate in this study.

Combination therapy using NPWT and ARS administration

The NPWT was initiated on the first day that wound dehiscence caused by an SSI was recognized, as described by Argenta and Morykwas [5]. A saline-soaked gauze dressing was placed into the wound cavity, a tube for fluid aspiration was placed on the dressing, and the dressing and the tube were sealed airtight with clear, adherent drape. Continuous aspiration was applied to the open wound. The pressure applied to the wound was 75 to 125 mmHg below ambient pressure. The NPWT was changed under an aseptic procedure every 2 to 3 d. The NPWT continued until the wound was closed completely.

The ARS, in addition to enteral nutrition, was administered on the first day that the wound dehiscence caused by an SSI was recognized in all patients, except for one who required additional time to obtain informed consent. The ARS was given to each patient three times per day orally, through a nasogastric tube, or through a gastrostomy. The supplement (Arginaid; Nestle Nutrition, Tokyo, Japan) included 2.5 g of arginine in one pack (125 mL). The ingredients in Arginaid are listed in Table 2. Three packs of this supplement are recommended for adult patients with pressure ulcer. The daily-use volume of this supplement in pediatric patients was not determined. Therefore, assuming that adults have an estimated body weight of 50 to 70 kg, the pediatric daily-use volume was calculated per body weight as 5.0 to 7.5 mL/kg. This supplement provided 100 to 150 mg · kg^{-1} · d^{-1} of arginine to the patients. The ARS administration was terminated on the day the wound dehiscence was completely closed.

Measurement of diseased area in wound dehiscence and blood plasma arginine concentration

The area was measured every week after the start of this combination therapy until the wound dehiscence was completely closed to determine the change in the wound area with time. In addition, blood samples were collected weekly for the analysis of plasma arginine to evaluate the chronologic change of the blood plasma arginine concentration using high-performance liquid chromatography after column fluorescence.

Results

Features of wound dehiscence and clinical outcome

Table 3 presents the characteristics of the wound dehiscence. The average onset of wound dehiscence caused by SSI occurred on postoperative day 8. The bacterial species associated with the SSI were *Pseudomonas aeruginosa*, *Klebsiella*, and *Staphylococcus* species, including methicillin-resistant *Staphylococcus aureus*. The mean area of wound dehiscence was 13.5 cm^2. The mean depth of wound dehiscence was 1.8 cm. The wound dehiscence reached the level of the muscular fascia in all patients. Two of six patients (patients numbered 3 and 4) showed a small fistula connecting to the gastrointestinal tract. Therefore, a large amount of digestive juice flowed out to the wound. The mean day of starting NPWT was day 9 after the operation. Similarly, the mean day of staring arginine administration was 10.8 postoperative days.

The clinical outcomes in patients are presented in Table 3. All patients showed a dramatic improvement in wound dehiscence. Figure 1 shows the chronologic change of wound dehiscence after the starting combination therapy. The local infection was decreased and a red granulation protruded both sides of the dehiscence site 1 to 2 wk after starting the combination therapy. In addition, the small fistula connecting the gastrointestinal tract in patient numbers 3 and 4 was closed 1 wk after the administration of this combination therapy. The dehiscence site gradually improved and closed within 1 mo after starting the combination therapy in all patients. The mean duration for complete closure of the wound dehiscence was 22.5 d after starting the combination therapy.

Table 2
Ingredients in Arginaid (one pack = 125 mL)

Calorie (kcal)	100
Protein (g)	5
Lipid (g)	0
Carbohydrate (g)	20
Water (mL)	107
Iron (mg)	7.0
Zinc (mg)	10
Copper (mg)	1.0
Selenium (g)	50
Vitamin A (g)	150
Vitamin D (g)	2.4
Vitamin E (mg)	5.0
Vitamin C (mg)	500
Vitamin B1 (mg)	0.7
Vitamin B2 (mg)	0.8
Niacin (mg)	10
Vitamin B2 (mg)	1.0
Folic acid (g)	100
Pantothenic acid (mg)	5
Arginine (mg)	2500