Table 1 Frequencies of LOH, LOI and MOI in human ovarian cancers.

(A) Ovarian cancer tissues	H19/ R sal	IGF2/ Apal	KCNQ1/ Smal	LIT1/ R sal	GTL2/ T aal	PEG1/ AFIII	PEG3/ MnlI	NDN/ MboI
Heterozygosity	58.5 (41/70)	47.1 (33/70)	55.5 (40/72)	16.2 (12/74)	58.3 (42/72)	50.0 (37/74)	34.2 (25/73)	41.8 (31/74)
LOH	4.8 (2/41)	9.0 (3/33)	5.0 (2/40)	0.0 (0/12)	7.1 (3/42)	8.1 (3/37)	4.0 (1/25)	0.0 (0/31)
LOI	29.2 (12/41)	45.4 (15/33)	12.5 (5/40)	16.6 (2/12)	23.8 (10/42)	45.9 (17/37)	8.0 (2/25)	6.4 (2/31)
MOI	56.0 (23/41)	33.3 (11/33)	77.5 (31/40)	83.3 (10/12)	66.6 (28/42)	45.9 (17/37)	84.0 (21/25)	93.5 (29/31)
ND	9.7 (4/41)	12.1 (4/33)	5.0 (2/40)	0.0 (0/12)	2.3 (1/42)	0.0 (0/37)	4.0 (1/25)	0.0 (0/31)
(B) Cell lines								
Heterozygosity	19.0 (4/21)	14.2 (3/21)	33.3 (7/21)	14.39 (3/21)	23.8 (5/21)	42.8 (9/21)	23.8 (5/21)	23.8 (5/21)
LOI	0/4	2/3	3/7	1/3	2/5	3/9	0/5	0/5
MOI	4/4	1/3	4/7	2/3	3/5	6/9	5/5	5/5

Percents of LOH (loss of heterozygosity), LOI (loss of imprinting) and MOI (maintenance of methylation) determined using RFLP analysis of 8 imprinted genes in 74 samples of ovarian cancers and 21 cell lines

significantly more hypermethylated than normal ovarian tissues (normal, 30.7% ± 15.1: HOC, 45.9% ± 15.5) (Table 3). The numbers of cancer tissue cases with hypermethylation above the range of the methylation rates in the normal ovarian surface epithelium were 17 for *H19*, 21 for *GTL2*, 21 for *ZDBF2*, and 14 for *PEG1* (Table 3). On the other hand, hypomethylation below the methylation level of normal ovarian tissues was found in 15 cases for *GTL2* and 23 for *ZDBF2*. We did not observe a significant difference in the DNA methylation between localized early-stage and advanced-stage tumor groups (Table 3). This suggested that the DNA methylation changes we detected occurred as early events of ovarian cancer.

The association between DNA methylation status and LOH/LOI in ovarian cancers

To determine whether the DNA methylation status in these DMRs of the imprinted genes acts as an indicator for potential LOH and/or LOI, we evaluated the

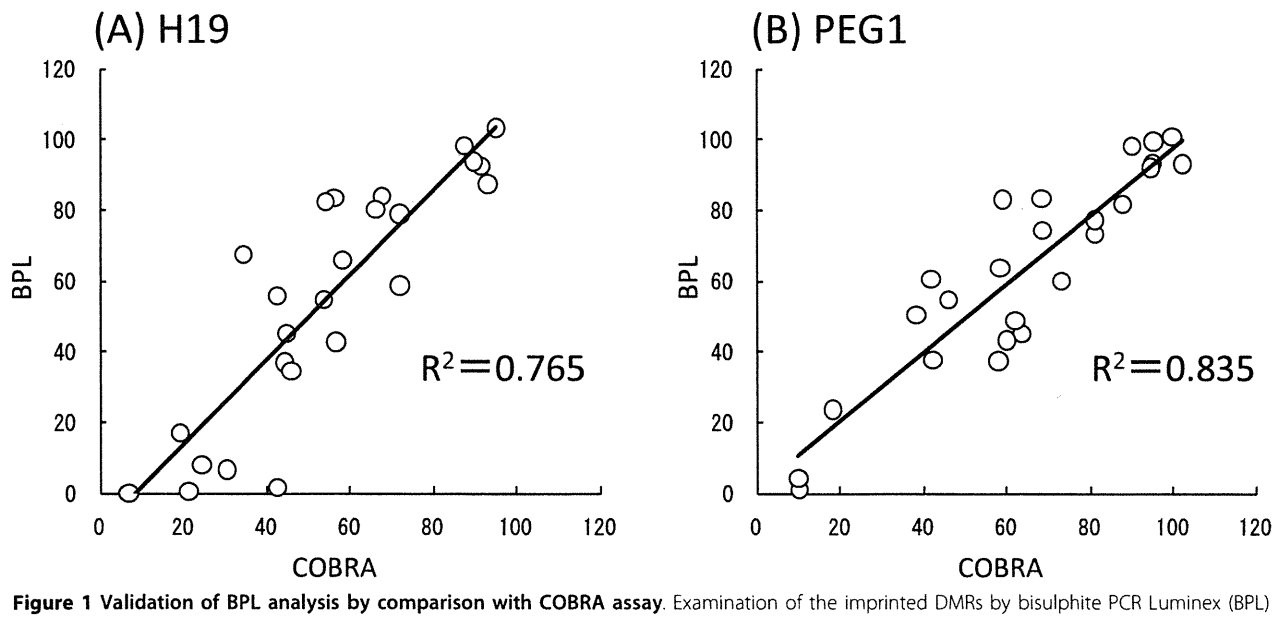

Figure 1 Validation of BPL analysis by comparison with COBRA assay. Examination of the imprinted DMRs by bisulphite PCR Luminex (BPL) and combined bisulphite PCR restriction analysis (COBRA) assay in DNA samples of ovarian cancer cell lines and normal cells. BPL: y-axis, COBRA: x-axis. Calculated by Spearman's rank method. *H19* (A) and *PEG1* (B).

Table 2 The methylation profiles of 8 imprinted genes by bisulphite PCR Luminex.

Cell lines	Histology	H19		GTL2		ZDBF2		PEG1		LIT1		ZAC		PEG3		SNRPN	
		BPL	COBRA	BPL	COBRA	BPL	COBRA	BPL	COBRA	BPL	COBRA	BPL	COBRA	BPL	COBRA	BPL	COBRA
		CpG (9,16)	CpG10	CpG (4,8)	CpG8	CpG (1,4,5)	CpG4	CpG15	CpG12	CpG (5,17,19)	CpG16	CpG8	CpG7	CpG20	CpG21	CpG19	CpG19
NC1	N	67.5	34.6	62.2	76.8	66.4	46.2	50.4	38.5	**42.7**	**27.4**	62.3	48.4	78.8	67.4	65.3	57.3
NC2	N	55.6	42.7	**67.3**	**82.1**	67.9	72.5	54.8	46.2	53.7	37.5	65.2	69.3	65.4	48.9	78.5	67.3
NC3	N	**65.8**	**58.3**	81.2	63.5	76.3	82.1	**45.3**	**63.9**	26.4	41.6	50.2	72.1	65.8	83.2	56.3	68.2
NC4	N	58.6	72.1	76.4	80.4	80.2	67.2	37.5	58.2	**35.8**	**54.7**	47.2	52.8	45.9	58.3	65.2	74.9
OC1	S	**10.32**	**95.4**	69.8	53.2	87.3	92.4	**83.1**	**59.2**	64.3	48.3	89.5	98.3	74.4	83.2	65.4	58.4
OC2	M	16.8	19.4	61.4	70.5	95.7	97.2	48.8	62.2	62.1	48.0	20.1	10.2	**92.1**	**89.5**	29.1	49.7
OC3	S	87.4	93.2	96.3	93.4	83.7	28.6	**98.3**	**90.4**	87.4	56.9	87.3	96.5	98.5	96.4	28.4	37.4
OC4	S	42.8	56.8	97.3	93.2	99.3	98.5	0.9	10.4	13.2	17.1	100.9	98.3	**48.7**	**47.2**	28.1	24.2
OC5	S	83.4	56.4	89.2	96.6	95.3	72.8	93.2	102.4	28.4	32.6	100.3	91.3	98.8	95.6	54.3	60.4
OC6	E	78.9	72.1	94.3	82.1	38.3	18.3	83.2	68.5	**34.2**	**56.2**	85.3	78.9	57.2	97.5	40.5	53.1
OC7	C	0.4	21.3	19.8	29.3	41.7	55.5	60.5	42.0	21.2	11.3	34.5	40.1	90.7	99.0	23.5	52.8
OC8	C	0.1	6.9	6.4	10.5	91.7	87.3	**81.8**	**88.1**	35.2	29.1	49.8	53.2	102.1	95.7	21.8	33.2
OC9	S	82.3	54.3	**37.4**	**65.2**	22.4	42.8	**23.6**	**18.5**	43.7	56.8	69.5	74.9	**68.5**	**90.2**	38.2	56.3
OC10	C	83.8	67.8	**78.4**	**65.9**	27.9	32.3	63.8	58.7	42.8	60.2	89.9	93.7	59.6	43.9	38.9	71.4
OC11	S	92.4	91.6	17.1	25.0	6.0	18.0	43.2	60.1	18.2	10.0	72.5	58.2	101.4	100.0	75.5	60.5
OC12	S	54.7	53.8	23.7	18.8	48.6	**74.3**	**68.9**	58.4	48.3	93.6	98.8	**67.4**	**39.8**	**73.4**	57.1	51.2
OC13	S	**80.3**	**66.2**	**42.3**	**40.1**	86.0	74.2	**93.4**	95.2	10.5	8.8	94.4	90.5	**102.1**	**100.0**	36.0	39.5
OC14	C	98.1	87.7	67.4	40.3	99.3	90.2	99.6	95.5	12.3	1.6	34.3	41.2	92.1	100.0	38.1	51.2
OC15	C	45.2	45.0	93.0	98.2	99.7	95.3	**92.1**	**94.8**	55.0	47.5	27.5	36.4	86.1	97.2	48.7	10.1
OC16	S	36.9	44.6	90.6	92.0	93.0	92.3	100.7	100.0	13.0	6.2	28.9	35.7	93.2	100.0	22.9	17.6
OC17	C	93.8	89.8	61.8	68.3	21.8	22.2	**37.9**	**42.5**	**48.4**	**31.8**	20.6	31.1	98.3	95.5	0.0	0.2
OC18	C	**34.4**	**46.0**	94.5	98.0	95.3	98.0	60.1	73.2	35.2	29.4	17.3	35.0	87.2	100.0	22.7	11.1
OC19	C	67	30.7	**47.8**	**62.1**	87.4	85.3	4.1	10.1	**11.2**	**0.0**	100.1	100.0	84.6	92.1	30.4	31.4
OC20	M	1.6	42.6	**58.1**	**60.3**	62.4	55.2	**73.4**	**81.4**	18.3	13.4	49.2	68.2	92.8	98.5	86.7	44.8
OC21	S	**8.0**	**24.5**	**80.2**	**87.0**	96.6	90.1	77.2	81.2	4.5	8.1	100.5	97.5	107.8	98.2	16.7	16.2
Normal ovarian surface tissues																	
NT1 (42)		57.5	76.2	43.4	51.2	37.2	65.4	51.4	38.6	**49.1**	**42.3**	53.3	41.2	42.1	40.2	521	47.8
NT2 (48)		**27.6**	**25.0**	23.6	21.6	46.1	34.5	20.9	34.4	23.1	12.2	5.1	32.3	21.3	20.6	22.1	33.3
NT3 (40)		61.5	31.8	**49.9**	**60.0**	50.4	45.0	31.0	42.8	17.1	10.9	48.8	29.4	21.0	33.6	24.3	34.8
NT4 (38)		28.9	25.0	**28.4**	**29.6**	46.1	39.3	**33.4**	**47.8**	28.8	17.7	67.4	46.1	45.6	33.8	80.5	29.0
NT5 (55)		**24.2**	**26.9**	**27.2**	**18.5**	33.2	29.0	**30.9**	**48.1**	**25.1**	**17.6**	10.7	44.5	75.5	40.0	32.3	31.3
NT6 (45)		**25.9**	**22.1**	**32.2**	**20.5**	46.4	62.5	60.0	40.4	24.1	17.3	25.2	23.4	44.2	69.0	77.4	40.9
NT7 (41)		34.6	24.6	**38.0**	**19.9**	49.9	37.4	27.3	46.2	**27.7**	**24.5**	21.0	63.1	49.7	53.7	25.6	24.5

Letters in blue and black boldface represent LOH, LOI and MOI, respectively. The Luminex value indicates the average value of DNA methylation

Table 3 Characterization of methylation profiles of the imprinted genes in DNA of ovarian cancers.

		H19	GTL2	ZDBF2	PEG1	LIT1	ZAC	PEG3	SNRPN
(A) Histology									
Normal	(n = 7)	34.3 ± 11.8	34.7 ± 9.5	44.2 ± 6.6	30.7 ± 15.1	27.9 ± 12.6	33.1 ± 5.8	42.8 ± 18.5	44.9 ± 25.3
Cancer	(n = 74)	41.7 ± 17.2	39.6 ± 18.8	43.8 ± 6.6	45.9 ± 15.5*	27.9 ± 14.1	41.1 ± 6.4	41.1 ± 14.1	42.8 ± 12.9
Serous	(n = 36)	47.6 ± 18.9	38.8 ± 22.0	42.0 ± 18.5	48.9 ± 14.7*	28.8 ± 9.8	42.7 ± 16.6	40.9 ± 14.8	39.3 ± 12.2
Mucinous	(n = 9)	36.0 ± 15.0	35.2 ± 19.2	36.8 ± 14.0	47.1 ± 20.0	34.7 ± 21.4	42.2 ± 5.9	31.2 ± 11.2	42.7 ± 17.8
Endometrioid	(n = 10)	37.9 ± 14.8	30.7 ± 14.4**	57.8 ± 19.8	45.5 ± 13.7	22.8 ± 13.0	37.5 ± 11.5	46.8 ± 15.9	41.4 ± 10.8
Clear	(n = 18)	45.6 ± 20.2	54.0 ± 20.1	39.7 ± 20.8	42.2 ± 13.8	25.5 ± 12.4	40.2 ± 16.1	45.7 ± 14.5	47.8 ± 11.1
(B) Progress (Staging)									
Localized (I, II)	(n = 29)	38.8 ± 14.9	40.8 ± 20.4	46.7 ± 19.4	47.8 ± 17.1	30.4 ± 16.1	41.0 ± 11.9	42.8 ± 17.8	44.2 ± 12.9
Advanced (III, IV)	(n = 45)	47.8 ± 19.6	41.3 ± 22.2	41.9 ± 19.2	45.9 ± 13.7	26.2 ± 10.0	41.1 ± 16.8	41.2 ± 12.8	40.9 ± 12.6
(C) Age									
Under 44 years	(n = 17)	46.7 ± 18.5	35.3 ± 21.0	42.0 ± 20.4	48.9 ± 13.5	24.2 ± 11.7	39.9 ± 12.3	44.7 ± 14.3	36.3 ± 10.7
45-55 years	(n = 29)	39.9 ± 14.7	47.2 ± 23.0	45.8 ± 17.5	46.9 ± 15.0	30.1 ± 14.8	38.9 ± 15.5	41.9 ± 15.8	45.4 ± 11.1
Over 56 years	(n = 32)	47.4 ± 21.2	38.4 ± 18.1	42.2 ± 21.1	45.1 ± 16.2	28.2 ± 10.9	44.1 ± 15.7	40.8 ± 14.9	42.5 ± 14.5

The values in the list are mean ± SD (standard deviation). Statistically significant differences between groups are presented as *P < 0.05, and **P < 0.01 by ANOVA

association between DNA methylation and LOH and/or LOI in the *IGF2/H19* and *PEG1* imprinted domains separately.

IGF2, which acts as a dominant oncogene, and *H19*, a physically and mechanistically linked gene on human chromosome 11, are reciprocally imprinted. In the paternal allele, *H19* DMR is methylated and silenced, whereas the reciprocally imprinted gene *IGF2* is transcribed. By contrast, in the maternal unmethylated allele, *H19* is expressed but *IGF2* is inactivated because of the binding of the repressor factor CTCF to the unmethylated *H19* DMR, which then prevents the *H19/IGF2* common enhancers from activating the *IGF2* promoter [19]. *IGF2* was found to have high frequencies of both LOH and LOI in HOC. *H19* was also found to have high frequencies of LOI (29.2%, 12/41). Nine of 14 cases with both *IGF2* and *H19* heterozygosity showed LOH or LOI of both genes and only one case had MOI for one of the two. Thus relaxation of *IGF2* and *H19* imprinting is frequent. In the *IGF2/H19* imprinted region, the samples with LOH and/or LOI at *H19* was more methylated than those with MOI (Figure 2A, Additional file 3: Table S2).

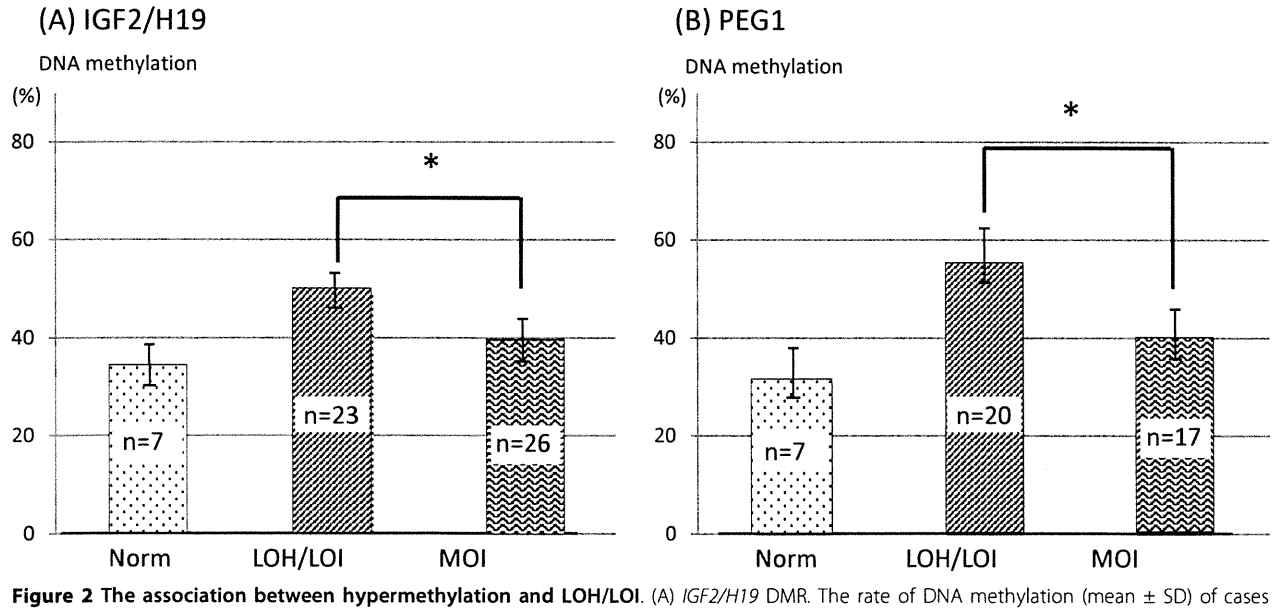

Figure 2 The association between hypermethylation and LOH/LOI. (A) *IGF2/H19* DMR. The rate of DNA methylation (mean ± SD) of cases with LOH/LOI of either *IGF2* or *H19* and MOI were compared. For this analysis, one case which *H19* was MOI and *IGF2* was LOI, was removed. (B) *PEG1* DMR. Statistically significant differences between groups are analyzed with ANOVA followed with multiple comparison method (Tukey's HSD test), presented as the *P*-value (*P < 0.01). Norm: Normal ovarian tissues.

Our results for *H19* were similar to a previously reported finding [20]. *PEG1* was reported to be a TSG and was also found to have high frequency of LOH/LOI in HOC. We also found that the samples with LOH/LOI at *PEG1* were more methylated than those with MOI with statistical significance (Figure 2B, Additional file 3: Table S2).

Discussion

Alterations in DNA methylation are the most common molecular alterations in human malignancies. Detection of the aberrant DNA methylation associated with cancer-related genes is a promising approach to improve cancer prevention, diagnosis and treatment options. Bisulphite modification is a prerequisite for most popular techniques aiming at detecting changes in methylation, but has been limited by throughput capacity. In this study, we used a high-throughput methylation detection method to analyze DNA methylation at 8 imprinted DMRs in epithelial ovarian cancer. We found that the PCR-Luminex method precisely quantified the methylation status of specific DNA regions in somatic cells and was also relatively rapid, economical and easy to use.

In the epithelial ovarian cancers, the frequency of LOI was higher than that of LOH. In particular, LOI was most frequent at *PEG1*, *IGF2* and *H19* DMRs. The frequency, extent of changes in DNA methylation and loci affected varied considerably among the samples. Generally, we found that DNA methylation at imprinted DMRs was increased in both cell lines and primary material. Importantly, we showed that gain of DNA methylation in the imprinted DMRs was apparent in tumors with LOI, especially at *PEG1* and *H19*. We also found DNA methylation changes in the absence of LOI. In other words, there were changes in DNA methylation at DMRs that were not associated with biallelic gene expression.

When we examined the clinical characteristics of the tumors, we found no significant differences in the frequency of LOI and aberrant DNA methylation between the localized early-stage and advanced-stage tumor groups. This suggested that the changes we identified occurred as a relatively early event of HOC. In general, the *PEG1* and *H19* DMRs appeared to be particularly prone to errors. This is similar to the previous findings in human sperm from subfertile men [21]. In *ZDBF2* and *GTL2* DMRs, aberrant DNA methylation occurred in HOC. As with *H19*, these DMRs are paternally methylated DMRs in somatic cells. In childhood cancers such as retinoblastoma, Wilms' tumor and osteosarcoma, changes primarily occur on the paternal allele first, followed by a second hit on the maternal allele [5,6,22]. Similarly, methylation of paternally imprinted DMR in normal somatic cells might be a first hit and cause ovarian carcinogenesis. These observations suggest a role for altered genomic imprinting in the malignant transformation process.

A previous report had demonstrated the association between the abnormal genomic imprinting of *H19* and *IGF2* expression [20]. The aberrant hypermethylation in the CTCF binding site of the *H19* gene was seen in the cases of HOC and correlated with *IGF2* LOI. Our results for *H19* were similar to those reported findings. The most frequent methylation error in HOC was seen in the *PEG1* DMR. In our previous report, we showed that demethylation of *PEG1* was present in growing oocytes from superovulated infertile women [23]. This *PEG1* DMR may be especially vulnerable to errors. LOI of PEG1 has subsequently also been implicated in the aetiology of lung adenocarcinomas, breast and colon cancer.

HOC is the leading cause of death from gynecologic malignancies because the majority of cases are not detected until the disease is well advanced. Our understanding of cancer as a clonal genetic disease has led to the identification of genetic alterations in many cancer types. However, ovarian cancer remains less well characterized. Only a few TSG genes acting in a recessive manner have been identified as somatically mutated or methylated in ovarian cancer, including *TP53* (48%) [24], *PTEN* (21% in the endometrioid subtype) [25], *RB1* (7-10%) [26], and *CDKN2A* (79% in the mucinous subtype) [27]. Biomarkers provide useful tools in screening for cancer and are now emerging as highly informative for monitoring disease status [28]. They can improve early detection and also the quality of life of patients with ovarian cancer. DNA methylation offers an additional tool that can be used in combination with other markers [29]. In addition, it has been established that DNA methylation biomarkers are present in patient serum and other body fluids [30]. To date, several methylated genes have been found to be highly prognostic for specific cancers, including those of the prostate [31], breast [32] and lung [33]. Although some methylated markers such as RASSF1A and GSTP1 have potential as prognostic indicators individually [34,35], 'methylation signature' panels could be much more informative [36] and accurate for monitoring cancer progression. Methylation patterns have previously been suggested to be tumor and stage specific [37]. Our work demonstrates that there is aberrant DNA methylation at several imprinted DMRs in HOC with changes at *PEG1* and *H19* being the most frequent and earliest alterations detected.

Conclusion

This is the first study reporting the use of PCR-Luminex for identification of prognostic panels of DNA methylation biomarkers for cancer. We believe that this approach is amenable to the classification of clinically relevant methylation patterns in a wide variety of tumors (and other pathologies) linked to the aberrant DNA methylation of imprinted DMRs. This BPL method may be sufficiently

sensitive that it can be applied to the analysis of DNA methylation in the very small number of circulating cancer cells found in blood and urine samples from patients.

Methods

Ovarian cancer cell lines and primary culture of surface epithelial cells

Twenty-one HOC cell lines were used in our study: 10 from serous adenocarcinoma (OVCAR3, CAOV3, JHOS2, HTOA, SKOV3, OV90, JHOS3, JHOS4, KF, MH), 2 from mucinous adenocarcinoma (OMC3, MCAS), 8 from clear cell adenocarcinoma (ES2, JHOC5, TOV21G, JHOC7, JHOC8, KM, HAC2, RMG) and 1 from endometrioid adenocarcinoma (TOV112D). The sources of these cells and culture methods were as described previously [38,39]. Four primary cultures of normal human ovarian surface epithelial (OSE1-4) cells were initiated from surface scrapings of normal ovaries as described [40].

Ovarian cancer tissue(s)

Seventy-four primary HOC tissues (36 serous, 9 mucinous, 10 endometrioid, 18 clear cell, and 1 other, Table 3) were obtained from patients presenting at our hospital. The mean ± standard deviation (SD) of the patients' ages for normal ovary and ovarian cancer tissues were 44.1 ± 6.6 and 52.9 ± 7.3, respectively. Seven specimens of normal ovarian surface epithelium were obtained from patients with benign non-ovarian disease. Histological diagnoses and clinical staging were performed according to the International Federation of Gynecologists and Obstetricians (FIGO) criteria. The numbers of cancer patients with localized tumors (stage I and II) and advanced tumor (stages III and IV) were 29 and 45, respectively. The samples were stained with hematoxylin and eosin to demonstrate > 85% of epithelial tumor cells. DNA and RNA were then extracted from the remaining samples [38]. DNA was also extracted from peripheral blood in matched patients. The study was performed after obtaining the patients' informed consent and with approval from the institutional ethics committee of the Tohoku University Graduate School of Medicine.

Analysis of loss of heterozygosity (LOH) and loss of imprinting (LOI)

PCR was performed on patient blood and tumor genomic DNA using the primer sequences summarized in Additional file 4: Table S3. A PCR reaction mix containing 0.5 μM of each primer set, 200 μM dNTPs, 1 × PCR buffer, and 1.25U of EX *Taq* Hot Start DNA Polymerase (Takara Bio, Tokyo, Japan) in a total volume of 20 μl was used. The following PCR program was used: 1 minute of denaturation at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C and a final extension for 5 minutes at 72°C. PCR products were digested by unique polymorphic enzymes to identify samples that were heterozygous for a single nucleotide polymorphism (SNP). For samples found to be heterozygous for a SNP, RNA was prepared from matched tumors, followed by reverse transcription-PCR (RT-PCR) and by restriction digestion [41-48]. The digested PCR products were electrophoresed on 2% agarose gel.

DNA methylation analysis

Bisulphite PCR-Luminex (BPL) methylation analysis was performed as described [18]. PCR primers sets, biotinylated at their 5'-end, were designed for gene amplification. PCR reaction mix contained 0.2 μM primer, 0.2 mM dNTPs, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3). 3 mM $MgCl_2$, 2% dimethyl sulfoxide (DMSO), 0.625U *Taq* DNA Polymerase (Roche, Tokyo, Japan) and 100-200 ng of bisulphite treated DNA in a total volume of 25 μl. PCR conditions: 40 cycles of 95°C for 20 s/60°C for 30 s/72°C for 30 s using a GeneAmp 9700 thermal cycler (Applied Biosystems, CA, USA). Oligonucleotide probe sequences (Additional file 2: Table S1 in Ref 18) were synthesized and covalently bound to carboxylated fluorescent microbeads using ethylene dichloride (EDC). These oligonucleotide-labeled microbeads (oligobeads) were mixed together to make an oligobeads mixture of 100 oligobeads/μl and hybridized to the 5'-biotin-labeled PCR amplicons in a total volume of 50 μl per well in a 96-well plate by adding 5 μl of the appropriate oligobead mixture and 5 μl of the PCR amplicons to 40 μl of hybridization buffer. This reaction mixture was first denatured at 95°C for 2 min and then hybridized at 48°C for 30 min. After hybridization, the oligobeads were washed in 100 μl of PBS-Tween and pelleted by microcentrifugation. Pelleted oligobeads were reacted with a 70 μl aliquot of a 100 × diluted solution of SA-PE in PBS-Tween. Hybridized amplicons were labeled with SA-PE at 48°C for 15 min. Reaction outcomes were measured by the Luminex 100 flow cytometer. Methylation assays were additionally performed for each DMR using the conventional bisulphite treatment PCR methylation assay and combined bisulphite PCR restriction analysis (COBRA) as described previously [21].

Statistical analyses

Differences between groups were analysed by analysis of variance, followed by Post-hoc, Tukey's HSD test. Statistical analyses were performed using the JMP (v9.0.0, SAS Institute Japan, Tokyo, Japan). Statistically significant differences between groups are presented as $^{*}P < 0.05$, and $^{**}P < 0.01$. Results for BPL and COBRA were compared using Spearman's rank method and Pearson's product-moment correlation coefficients.

Additional material

Additional file 1: Table S1 The list of LOH, LOI and MOI in HOC. LOH, LOI and MOI determined using RFLP analysis of 8 imprinted genes are summarized. NC: Normal cells (NC1-4). NT: Normal ovarian tissues (NT1-7). CC: Cancer cell lines (CC1-21). CT: Cancer tissue (CT1-74).

Additional file 2: Figure S1 Validation of BPL analyses by comparison with COBRA assay. Examination of the imprinted DMRs by bisulphite PCR Luminex (BPL) and combined bisulphite PCR restriction analysis (COBRA) assay in DNA samples of ovarian cancer cell lines and normal cells. BPL: y-axis, COBRA: x-axis. The number was calculated by Spearman's rank method. *GTL2* (C), *ZDBF2* (D), *LIT1* (E), *ZAC* (F), *PEG3* (G) and *SNRPN* (H).

Additional file 3: Table S2 Sequences of PCR primers and restriction enzymes used for PCR-RFLP analysis.

Additional file 4: Table S3 Bisulphite PCR-Luminex and COBRA methylation profiles of the eight imprinted DMRs in the DNA of human ovarian cancer cells and normal ovarian tissues. Numbers in blue and black boldface indicate LOI and MOI, respectively. Luminex values indicate average methylation values at the sites tested. NC: Normal cells (OSE1, OSE2, OSE3, OSE4). CC: Cancer cell lines (CC1: OVCAR3, CC2: OMC3, CC3: CAOV3, CC4: JHOS2, CC5: HTOA, CC6: TOV112D, CC7: ES2, CC8: JHOC5, CC9: SKOV3, CC10: TOV21G, CC11: OV90, CC12: JHOS3, CC13: JHOS4, CC14: JHOC7, CC15: JHOC8, CC16: KF, CC17: KM, CC18: HAC, CC19: RMG, CC20: MCAS, CC21: MH. NT: Normal ovarian tissues (NT1-NT7). N: Normal, S: Serous adenocarcinoma, M: Mucinous adenocarcinoma, E: Endometrioid carcinoma, C: Clear cell carcinoma.

Abbreviations

BPL: Bisulphite PCR-Luminex; COBRA: Combined bisulphite PCR restriction analysis; DMR: Differentially methylated region; FIGO: International Federation of Gynecologists and Obstetricians; HOC: Human ovarian cancer; LOH: Loss of heterozygosity; LOI: Loss of imprinting; MOI: Maintenance of imprint; ND: Not determined; PCR: Polymerase chain reaction; QOL: Quality of Life; RFLP: Restriction fragment length polymorphism; RT-PCR: Reverse transcription-PCR; SNP: Single nucleotide polymorphism.

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Authors' contributions

HH, HO, HK, NM, FS and AS performed the DNA methylation analyses and validation of the BML method. FS, SN, JS, NY carried out the product and culture of cancer cell lines and collect the tumor samples. KN did the statistical analyses. HH and TA wrote this manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Case report

Liver-specific mitochondrial respiratory chain complex I deficiency in fatal influenza encephalopathy

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Abstract

We report on a 4-year-old boy who died from influenza encephalopathy. The clinical course and microscopic findings of the autopsied liver were compatible with Reye's syndrome. We examined the mitochondrial respiratory chain function by blue native polyacrylamide gel electrophoresis (BN-PAGE), western blotting, and respiratory chain enzyme activity assays. The activity of liver respiratory chain complex (CO) I was markedly decreased (7.2% of the respective control activity); whereas, the other respiratory chain complex activities were substantially normal (CO II, 57.9%; CO III, 122.3%; CO IV, 161.0%). The activities of CO I–IV in fibroblasts were normal (CO I, 82.0%; CO II, 83.1%; CO III, 72.9%; CO IV, 97.3%). The patient was diagnosed with liver-specific complex I deficiency. This inborn disorder may have contributed to the fatal outcome. We propose that relying only on fibroblast respiratory chain complex activities may lead to the misdiagnosis of liver-specific complex I deficiency.

Keywords: Influenza encephalopathy; Reye's syndrome; Mitochondria; Complex I deficiency; Liver-specific

1. Introduction

Influenza encephalopathy is a critical complication of influenza infection. Although the pathological mechanism is poorly understood, mitochondrial malfunction is suggested to play a role in the pathogenesis [1]. We describe a boy with liver-specific mitochondrial respiratory chain complex I deficiency who developed fatal encephalopathy associated with influenza A infection. The possible contribution of the mitochondrial respiratory chain disorder to the clinical course is discussed.

2. Case report

A 4-year-old Japanese boy developed pyrexia. He was treated with acetaminophen once and visited the family doctor. Influenza A infection was diagnosed by nasal antigen test in a clinic and he was treated with oseltamivir. He was admitted to a nearby hospital due to a generalized seizure in the evening; then, he was transferred to our institute because of highly elevated serum transaminase. He was the first child born to healthy parents with no consanguinity. No other child had died in early

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infancy within three degrees of relationship. He had normal psychomotor development and had not been vaccinated against influenza.

On arrival, he was comatose and had a temperature of 38.9 °C, heart rate of 136 beats per minute, and blood pressure of 106/62 mm Hg. Neither arrhythmia nor cardiac hypertrophy was seen in the electrocardiogram or echocardiography. Blood examination showed marked liver dysfunction and ammonemia (aspartate aminotransferase, 4282 IU/l; alanine aminotransferase, 1750 IU/l; ammonia, 156 μg/dl). Blood gas analysis showed marked acidosis (pH 6.964, pCO_2 59.6 mm Hg, HCO_3 11.2 mol/l, BE −23.7 mmol, and lactate 9.0 mmol/l). Blood glucose was 128 mg/dl under intravenous infusion. Influenza encephalopathy was diagnosed and intensive therapy, including mechanical ventilation, steroid, and heart stimulants, was started. A few hours later, he developed cardio-pulmonary arrest and died 36 h after developing pyrexia. This clinical course led us to suspect Reye's syndrome and mitochondrial disorders. The parents consented to resection of the patient's liver and skin fibroblasts. Urine organic acid analysis, blood amino acid profile, and carnitine profile did not show any findings suggestive of congenital metabolic disorders. Microscopical finding showed microvesicular fatty droplets in hepatic cytoplasm in hematoxylin-eosin and oil red O staining (Fig. 1), that was compatible with Reye's syndrome. The grade of histological hepatic changes was milder than the fulminant clinical course.

The activities of respiratory chain complexes (Co) I, II, III, and IV were assayed in the crude post-600 g supernatant of the liver and in isolated mitochondria from skin fibroblasts as described previously [2]. The activity of each complex was presented as a percent ratio relative to the mean value obtained from 12 healthy controls. The activities of Co I, II, III, and IV were also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker, or Co II activity [2]. Liver respiratory chain complex I activities were very low, but CS, Co II, III, and IV activities were normal. In contrast to the liver, the fibroblast complex I activity was normal (Table 1).

The expression of the mitochondrial respiratory chain Co I, II, III, and IV proteins in the liver and fibroblasts were examined by Western blotting using blue native polyacrylamide gel electrophoresis (BN-PAGE) according to methods described previously [3]. The results of BN-PAGE are shown in Fig. 2. The band corresponding to Co I was not visible; while, the intensities of the Co II, III, and IV bands remained normal. Several base substitutions were detected by polymerase chain reaction, but there was no pathogenic mutation in the genomic DNA extracted from the autopsied liver tissue.

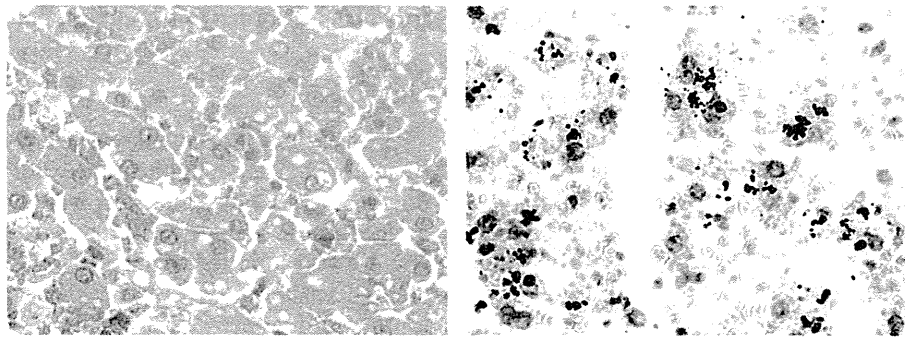
3. Discussion

Mitochondrial malfunction has been described in influenza encephalopathy. There are no reports of mitochondrial respiratory chain diseases, although disorders of fatty acid oxidation have been discussed [1]. Complex I deficiency was first recognized in 1979 by Morgan-

Table 1
Enzyme assay of respiratory chain complexes.

%	Co I	Co II	Co III	Co IV	CS
Liver					
% of normal	7.2	57.9	122.3	161.0	78.1
CS ratio	9.2	74.1	155.0	203.8	–
Co II ratio	12.3	–	212.2	272.2	–
Fibroblast					
% of normal	82.0	83.1	72.9	97.3	120.4
CS ratio	66.2	66.8	56.5	76.3	–
Co II ratio	98.2	–	83.7	112.5	–

Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthase.
Enzyme activities are expressed as a % of the mean relative activity of the normal control and relative to CS and Co II.



A. Hematoxylin-Eosin staining (× 400) B. Oil Red O staining (× 400)

Fig. 1. Autopsy liver samples show preserved hepatic architecture with scattered distribution of micro-vesicular fatty droplets in the hepatic cytoplasm (A). Marked congestion, focal necrosis, and mild inflammatory cellular infiltration without fibrosis were noted. Fat deposition was also suggested with oil red O staining (B). The grade of histological hepatic changes was milder than the fulminant clinical course.

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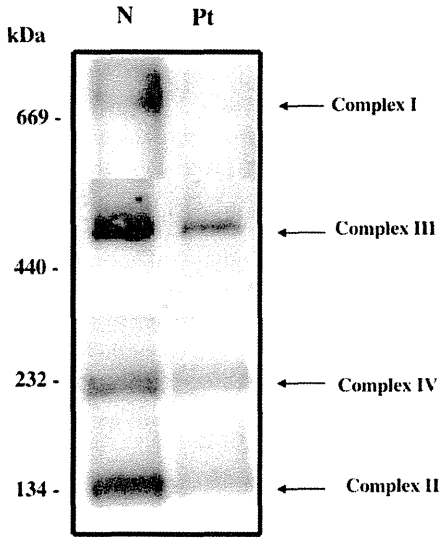

Fig. 2. Blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis of liver respiratory chain enzymes showed markedly decreased protein expression of complex I, while the protein bands of complex II, III, and IV were comparable to the control (N) samples.

Hughes; yet, studies have not progressed because of technical difficulties. More recently, complex I deficiency was regarded as the most common energy generation disorder. The manifestations range from typical mitochondrial diseases, such as Leigh syndrome, to obscure conditions such as slow regression or intractable secretory diarrhea [4].

Complex II activity has been shown to be more labile than complex I when measuring respiratory chain enzymes in patients with a wide range of metabolic disorders, liver failure, or liver disease [5]. In the present case, only complex I activity was very low; this indicates primary complex I deficiency rather than a secondary effect of influenza A infection. Complex I includes seven mitochondrial DNA-encoded subunits and at least 39 nuclear-encoded subunits. In our case, no mutation was detected in the mitochondrial DNA (mtDNA). The detection rate for mutations in mitochondrial or nuclear DNA in complex I deficiency is as small as 20% [6,7].

In the present case, complex I was deficient only in the liver, not in fibroblasts. Mitochondrial respiratory complex disorders can show clinical and biochemical tissue specificity [2,4,6,8,10]. For this reason, it is difficult to diagnose by suspension cells or serum enzyme assays. The possible mechanisms of tissue specificity are tissue-specific subunits of complex I [9], the ratio between normal and mutant mtDNA in a specific tissue [7], and tissue differences in RNA processing [10]. To our knowledge, very few cases with liver-specific complex I deficiency have been reported [2,8]. These reported cases had chronic neurological symptoms such as epilepsy, hypotonia, or developmental regression, with the exception of one case that had severe cardiomyopathy in early infancy [2]. There was one case without evidence of liver dysfunction [8]. Clinically there was no definite difference from usual Co I deficiency. One reason for the small number of cases is that the liver is not the prime diagnostic tissue. Respiratory chain complex deficiency is usually confirmed by tissue biopsy. Muscle is usually the prime diagnostic tissue, and cultured skin fibroblasts are also often analyzed [10]. False-negative diagnostic results may occur because the liver is not examined.

This case was determined to be complex I deficiency by BN-PAGE Western blotting and determination of enzyme activities. This is the first report of respiratory chain complex I deficiency in influenza encephalopathy. We suggest there may be many undiagnosed cases of this metabolic disorder. Here, we described a healthy child, who had never been suspected of having any disease, diagnosed with a metabolic disorder after acute encephalopathy with subsequent death. Future studies are needed to focus on the development of a method to detect this inborn metabolic disorder before onset.

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CLINICAL STUDY

Analysis of plasma ghrelin in patients with medium-chain acyl-CoA dehydrogenase deficiency and glutaric aciduria type II

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Abstract

Objective: Ghrelin requires a fatty acid modification for binding to the GH secretagogue receptor. Acylation of the Ser3 residue of ghrelin is essential for its biological activities. We hypothesized that acyl-CoA is the fatty acid substrate for ghrelin acylation. Because serum octanoyl-CoA levels are altered by fatty acid oxidation disorders, we examined circulating ghrelin levels in affected patients.
Materials and methods: Blood levels of acyl (A) and des-acyl (D) forms of ghrelin and acylcarnitine of patients with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency and glutaric aciduria type II (GA2) were measured.
Results: Plasma acyl ghrelin levels and A/D ratios increased in patients with MCAD deficiency or GA2 when compared with normal subjects. Reverse-phase HPLC confirmed that *n*-octanoylated ghrelin levels were elevated in these patients.
Conclusion: Changing serum medium-chain acylcarnitine levels may affect circulating acyl ghrelin levels, suggesting that acyl-CoA is the substrate for ghrelin acylation.

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Introduction

Ghrelin, an endogenous ligand for the GH secretagogue receptor, is an acylated peptide produced by gastrointestinal endocrine cells (1). Ghrelin is the only peptide known to require a fatty acid modification. Octanoylation of the Ser3 residue is essential for ghrelin-mediated stimulation of GH secretion and regulation of energy homeostasis via increased food intake and adiposity (2, 3). Other than octanoylation (C8:0), the hormone is subject to other types of acyl modification, decanoylation (C10:0), and possibly decenoylation (C10:1) (4, 5). Recently, ghrelin *O*-acyltransferase (GOAT), which octanoylates ghrelin, was identified (6, 7). The fatty acid substrate that contributes to ghrelin acylation, however, has not been clarified, although the presumed donor is acyl-CoA.

Mitochondrial fatty acid oxidation (FAO) disorders result from genetic defects in transport proteins or enzymes involved in fatty acid β-oxidation (8, 9). The clinical phenotypes have recently been associated with a growing number of disorders, such as Reye syndrome, sudden infant death syndrome, cyclic vomiting syndrome, fulminant liver disease, and maternal complications during pregnancy (10). Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, the most common inherited defect in FAO, causes elevated serum octanoylcarnitine levels (11), reflecting elevated octanoyl-CoA levels. Glutaric aciduria type II (GA2), which is caused by defects in electron transfer flavoprotein (ETF), ETF-ubiquinone oxidoreductase, or other unknown abnormalities in flavin metabolism or transport, is characterized by elevated serum acylcarnitine levels, including octanoylcarnitine (8, 9). In carnitine palmitoyltransferase II (CPT II) deficiency and very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, serum octanoyl-CoA levels do not increase, but at times actually decrease (8, 9).

We hypothesized that octanoyl-CoA is the fatty acid substrate for ghrelin acylation. To examine this hypothesis, we measured circulating ghrelin levels in patients with MCAD deficiency (MCADD) and GA2.

Materials and methods

Subjects

Five female patients with FAO deficiency (two with MCADD one with GA2, one with CPT II deficiency (12),

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and one with VLCAD deficiency) were recruited for this study. The study protocol was approved by the ethics committee on human research at the Kyoto University Graduate School of Medicine. Written informed consent was obtained prior to enrollment.

Measurement of plasma ghrelin concentrations

Because FAO patients tend to develop hypoglycemia by fasting, it was difficult to do overnight fasting. Therefore, blood samples for ghrelin analyses were drawn from a forearm vein in the morning after fasting as long as possible. Plasma samples were prepared as described previously (13). Blood samples were immediately transferred to chilled polypropylene tubes containing Na_2EDTA (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Kyoto, Japan: 1000 kallikrein inactivator units/ml = 23.6 nmol/ml (23.6 pM)) and centrifuged at 4 °C. One-tenth volume of 1 M HCl was immediately added to the separated plasma. The acylated and desacylated forms of ghrelin were measured using a fluorescence enzyme immunoassay (FEIA; Tosoh Corp. Tokyo, Japan). The minimal detection limits for acyl and des-acyl ghrelin in this assay system were 2.5 and 10 fmol/ml respectively. The interassay coefficients of variation were 2.9 and 3.1% for acyl and des-acyl ghrelins respectively.

Reverse-phase HPLC

Reverse-phase HPLC (RP-HPLC) was performed as described previously (4, 5, 14). Briefly, plasma diluted 50% with 0.9% saline was applied to a Sep-Pak C18 cartridge pre-equilibrated with 0.9% saline. The cartridge was washed with saline and 10% acetonitrile (CH_3CN) solution containing 0.1% trifluoroacetic acid (TFA). Adsorbed peptides were eluted with 60% CH_3CN solution containing 0.1% TFA. The eluate was evaporated and separated by RP-HPLC. All HPLC fractions were quantified using RIAs for ghrelin (4, 14, 15, 16). RIAs for a ghrelin C-terminal region (C-RIA) and a ghrelin N-terminal region (N-RIA) measure des-acyl ghrelin and octanoyl-ghrelin respectively (15). A RIA for N-terminal ghrelin showed ~20–25% cross-reactivity values for the *n*-decanoylated and *n*-decenoylated forms (16). Authentic human ghrelin-(1–28) was chromatographed with the same HPLC system.

Tandem mass spectrometry

Acylcarnitines in sera and dried blood spots were measured according to previously reported methods (17, 18), without derivatization. Briefly, 3 μl serum and 110 μl methanol solutions (99%) with deuterium-labeled acylcarnitines as internal standards were mixed and centrifuged, and 5 μl of the supernatant was introduced into liquid chromatography flow of methanol/acetonitrile/water (4:4:2) with 0.05% formic acid using a SIL-20AC autoinjector (Shimadzu, Kyoto, Japan). Flow injection and electrospray ionization tandem mass spectrometric (MS/MS) analyses were performed using an API 4000 LC/MS/MS system (AB Sciex, Tokyo, Japan). Positive ion MS/MS analysis was performed in precursor ion scan mode with an m/z value of 85 for the product ion. Data were recorded for 0.7 min after every sample injection and the recorded intensities of the designated ions were averaged using Chemoview Software (Foster City, CA, USA). All samples were measured serially within 1 day.

Results

We measured plasma ghrelin concentrations in patients with MCADD and GA2 (Table 1) and also in patients with CPT II and VLCAD deficiency. Elevated C8-acylcarnitine serum levels were observed in MCADD and GA II, whereas they were unchanged or lower in CPT II or VLCAD deficiency (Table 1). Levels of acyl ghrelin but not des-acyl ghrelin appeared to be elevated in patients with MCADD or GA2 in comparison with those in patients with CPT II or VLCAD deficiency, or those in female normal subjects from a previous study.

We then performed RP-HPLC analysis of ghrelin using plasma from patient 1 with MCADD. It demonstrated an eluted peak that corresponded to *n*-octanoylated human ghrelin-(1–28) in an N-RIA and a C-RIA, indicating that the detected acyl ghrelin was octanoylated (Fig. 1A). When plasma from patient 3 with GA2 was examined using the same method, the N-RIA revealed that the major peak corresponded to *n*-octanoylated human ghrelin-(1–28) (Fig. 1B). In addition, a small peak, which corresponded to decanoylated ghrelin, was observed in fraction 16 (arrow c), reflecting that serum C10-acylcarnitine levels were also elevated in patient 3 (Table 1).

Discussion

Ghrelin is the sole peptide hormone known to have a fatty acid modification. When we started this study in 2007, the catalytic enzyme and fatty acid substrate that mediate ghrelin acylation had not been identified. During this study, the GOAT enzyme was shown to be essential for ghrelin acylation (6, 7). Octanoic acid and octanoyl-CoA were candidates for the fatty acid substrate. We hypothesized that octanoyl-CoA was the substrate, because acylation of ghrelin should be an intracellular process. In fact, Ohgusu *et al.* (19) showed that acyl-CoA can be the substrate for ghrelin acyl-modification using the *in vitro* assay system. We tested this hypothesis in patients with MCADD and GA2,

Table 1 Clinical features, serum acylcarnitine levels, and plasma ghrelin concentrations in female patients with FAO disorders.

					Acylcarnitine (nmol/ml)										
Subjects	Disease	Age (years)	BMI	Height (cm)	C4	C6	C8	C10:1	C10	C12	C14	C16	C18	AG (fmol/ml)	DAG (fmol/ml)	A/D ratio
Patients ($n=5$)																
1	MCAD	6	15.1	119.5	0.30	0.55	4.61	0.95	0.29	0.04	0.01	0.05	0.01	45.09	57.23	0.79
2	MCAD	11	16.0	125.3	0.07	0.36	2.26	0.40	0.20	0.02	0.02	0.07	0.01	30.11	40.83	0.74
3	GA2	6	15.8	116.1	0.39	0.31	1.24	0.32	1.86	0.35	0.12	0.16	0.05	56.55	50.80	1.11
4	CPT II def.	10	17.8	141.5	0.10	0.06	0.21	0.20	0.40	0.15	0.03	0.08	0.02	19.76	34.49	0.57
5	VLCAD def.	5	14.8	109.5	0.07	0.09	0.07	0.08	0.28	0.42	2.17	2.00	0.87	27.02	113.07	0.24
Normal subjects ($n=20$; mean ± s.d.)[a]		32.6 ± 10.3	20.3 ± 1.9											19.66 ± 11.26	47.71 ± 43.71	0.48 ± 0.17
Reference range ($n=34$; mean ± s.d.)					0.25 ± 0.09	0.04 ± 0.02	0.07 ± 0.06	0.08 ± 0.05	0.13 ± 0.12	0.06 ± 0.05	0.03 ± 0.02	0.09 ± 0.04	0.04 ± 0.02			

C8, octanoyl acylcarnitine; C10, decanoyl acylcarnitine; C10:1, decenoyl acylcarnitine; AG, acyl ghrelin; DAG, des-acyl ghrelin; def., deficiency.
[a]See reference 13. All samples were reassayed using the FEIA.

which are characterized by higher intracellular octanoyl-CoA levels. Indeed, plasma A/D ratios tended to be elevated in these FAO deficiencies. A relationship between age and ghrelin levels may exist (20, 21). Concerning children, Ikezaki reported that the circulating ghrelin levels tended to correlate negatively with age in children and adolescents, but the correlation was not significant (22). Thus, the relationship has not been confirmed yet. Although we did not compare them directly with those in age- and body mass index (BMI)-matched normal children, they appeared to be higher than those in children with CPT II and VLCAD deficiencies with similar BMIs. BMIs of these patients were comparable to those of normal Japanese female children (23). These findings support the hypothesis that octanoyl-CoA is a primary substrate for ghrelin, although medium-chain triglyceride dietary lipids are a direct source for ghrelin acylation (7, 16, 24). Moreover, GOAT is a membrane-bound molecule in the endoplasmic reticulum (ER). Although how octanoyl-CoA gets into the ER lumen is unclear, Yang *et al.* (6) speculated that GOAT might mediate the transfer of octanoyl-CoA from the cytosol to the ER lumen. Although serum acylcarnitine levels tended to correlate with acyl ghrelin levels, further studies using more patients with FAO disorders are needed to confirm this relationship.

In addition to *n*-octanoylated ghrelin, other molecular forms of the ghrelin peptide exist, including des-acyl ghrelin lacking an acyl modification and such minor acylated ghrelin species as *n*-decanoylated ghrelin (Ser3 is modified by *n*-decanoic acid) (4, 5). Serum from a patient with GA2 showed the presence of acylated ghrelin that was not octanoylated and was possibly decanoylated (16). In a patient with GA2, intracellular levels of a variety of acyl-CoAs, including octanoyl- and decanoyl-CoAs, were increased, whereas MCADD was associated with specific elevation of octanoyl-CoA levels. In fact, the patient with GA2 had elevated octanoylcarnitine and decanoylcarnitine levels: 1.24 and 1.86 nmol/ml respectively. Nonetheless, the HPLC peak representing *n*-decanoylated ghrelin was much smaller than that representing *n*-octanoylated ghrelin. Although this is possibly because GOAT acylates ghrelin more efficiently with octanoyl-CoA than decanoyl-CoA, it is more likely because the cross-reactivity between *n*-octanoylated and *n*-decanoylated ghrelins is 20–25% in the N-RIA. In fact, the HPLC peaks of fraction 15–17 in the C-RIA, which detects similarly both *n*-octanoylated and *n*-decanoylated ghrelins, were large, strongly suggesting that a substantial amount of *n*-decanoylated ghrelin comparable to the elevated decanoylcarnitine level was present. Our observation that acyl ghrelin levels were not elevated in VLCAD and CPT II deficiencies, in which medium-chain acyl-CoAs levels are not higher, supported the idea that GOAT specifically acts on medium-chain acyl-CoAs. Although C16 and C18 levels were not increased in the patient with CPT II deficiency (Table 1), they may be normalized during

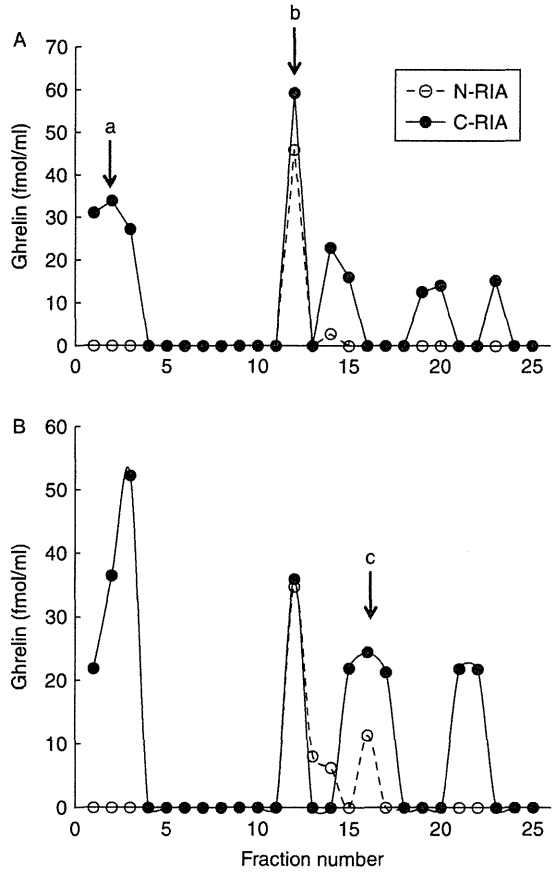

Figure 1 Representative RP-HPLC profiles of ghrelin immunoreactivity in patients with MCADD (A) and GA2 (B). Closed circles, data obtained using a RIA for a ghrelin C-terminal region (C-RIA); open circles, data obtained using a RIA for a ghrelin N-terminal region (N-RIA). Patient plasma extracts from a Sep-Pak C18 cartridge were fractionated using a Symmetry300 C18 column (5 mm packing, 3.9×150 mm, Waters). A linear gradient of 10–60% CH_3CN containing 0.1% TFA was passed over the column for 40 min at 1.0 ml/min. The fraction volume was 1.0 ml. Arrows indicate the elution positions of des-acyl human ghrelin-(1–28) (a), *n*-octanoylated human ghrelin-(1–28) (b), and *n*-decanoylated ghrelin (c).

a stable period in a mild form of CPT II deficiency (25). In fact, this patient did not manifest any marked signs or symptoms at the measurement.

Ghrelin modification with the fatty acid is essential for its biological action. Octanoylation of ghrelin may also be linked to energy homeostasis and fat metabolism. For instance, when serum *n*-octanoic acid levels increase following fat degradation, ghrelin octanoylation is enhanced, resulting in stimulation of fat synthesis. Thus, ghrelin may play an important role in energy homeostasis through its own fatty acid metabolism. Related to this concept, Kirchner *et al.* (24) speculated that signaling via GOAT and ghrelin might act as a fat sensor for exogenous nutrients and support fat storage as nutrients are ingested.

FAO deficiency contributes to such clinical problems as sudden infant death syndrome, cyclic vomiting syndrome, fulminant liver disease, and maternal complications (8, 9). Early diagnosis and appropriate management are required to reduce mortality and morbidity associated with this class of disorders. Recently, newborn screening has been expanded in this area. Measuring plasma ghrelin levels may support a diagnosis of MCADD or GA2, for example. Moreover, our results have pathophysiological implications for these disorders. Plasma ghrelin levels are changed by energy demands and food intake (e.g. glucose and fat), and ghrelin affects appetite and adiposity (2, 3). Alterations of plasma ghrelin levels in FAO disorders may reflect and/or influence the patient's metabolic status. In addition, higher acyl ghrelin levels may affect the GH/insulin-like growth factor 1 (IGF1) system. There are reports that higher AG levels would increase GH and IGF1 levels (26, 27, 28, 29) and thereby linear growth could be affected. Although none of our patients manifested markedly abnormal growth velocity, we did not measure their serum GH/IGF1 levels. Thus, further studies are warranted to detail a variety of metabolic parameters in this setting.

There are several limitations in this study. At first, the number of FAO patients tested is small. Unfortunately, the incidence of FAO patients in the Japanese population is much smaller than that in Caucasians. Although we asked pediatricians on a nationwide scale, we could successfully collect only five female patients. No adult case has yet been reported in Japan. Secondly, as mentioned above, the normal female subjects were not matched in age or BMI, although patients with MCADD and GA2 exhibited higher plasma A/D ratios than those in child CPT II and VLCAD deficiencies with similar BMIs. To supplement the correlation study, we performed RP-HPLC analysis to prove the increased octanoylation of ghrelin in MCADD and GA2 directly. Further, the presence of *n*-decanoylated ghrelin is also demonstrated in GA2. Thirdly, the disturbance in the hepatic carbohydrate regulation and the altered peripheral glucose uptake may occur in FAO patients. Hence, abnormal carbohydrate regulation could influence acyl ghrelin levels. Since none of our patients manifested abnormal fasting glucose and HbA1c levels, we speculated that no significant effects occurred.

In summary, we have demonstrated increased levels of acyl ghrelin in patients with MCADD or GA2, which are also characterized by increased intracellular octanoyl-CoA levels. These findings provide mechanistic insights into the biosynthesis of ghrelin. Furthermore, analyzing plasma ghrelin levels may help elucidate pathophysiological processes in FAO deficiencies and aid in the diagnosis of these disorders. Detailed studies using more patients are certainly needed.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy: Diagnosis of mitochondrial respiratory chain disorders

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ABSTRACT

Mitochondrial respiratory chain disorders are the most common disorders among inherited metabolic disorders. However, there are few published reports regarding the relationship between mitochondrial respiratory chain disorders and sudden unexpected death in infancy. In the present study, we performed metabolic autopsy in 13 Japanese cases of sudden unexpected death in infancy. We performed fat staining of liver and postmortem acylcarnitine analysis. In addition, we analyzed mitochondrial respiratory chain enzyme activity in frozen organs as well as in postmortem cultured fibroblasts. In heart, 11 cases of complex I activity met the major criteria and one case of complex I activity met the minor criteria. In liver, three cases of complex I activity met the major criteria and four cases of complex I activity met the minor criteria. However, these specimens are susceptible to postmortem changes and, therefore, correct enzyme analysis is hard to be performed. In cultured fibroblasts, only one case of complex I activity met the major criteria and one case of complex I activity met the minor criteria. Cultured fibroblasts are not affected by postmortem changes and, therefore, reflect premortem information more accurately. These cases might not have been identified without postmortem cultured fibroblasts. In conclusion, we detected one probable case and one possible case of mitochondrial respiratory chain disorders among 13 Japanese cases of sudden unexpected death in infancy. Mitochondrial respiratory chain disorders are one of the important inherited metabolic disorders causing sudden unexpected death in infancy. We advocate metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy cases.

1. Introduction

Sudden unexpected death in infancy (SUDI) is defined as sudden unexpected death occurring before 12 months of age. If SUDI remains unexplained after thorough investigations, it is classified as sudden infant death syndrome (SIDS). The more common causes of SUDI are infection, cardiovascular anomaly, child abuse, and metabolic disorders. However, the many potential inherited metabolic disorders are more difficult to diagnose at autopsy as compared to cardiovascular defects and serious infection. Inherited metabolic disorders may, therefore, be underdiagnosed as a cause of SUDI or misdiagnosed as SIDS. Fatty acid oxidation disorders (FAODs) are one type of the inherited metabolic disorders and may cause as much as 5% of SUDI cases after thorough investigations including metabolic autopsy [1–5]. In a review of SUDI cases with respect to potential FAODs, we found a case of carnitine palmitoyltransferase II deficiency [6]. In that study, we performed fat staining of liver, postmortem acylcarnitine analysis, and genetic analysis, advocating the importance of metabolic autopsy in SUDI cases.

Mitochondrial respiratory chain (MRC) disorders were first identified in 1962 [7]. MRC disorders have a frequency of about at least 1:5000 newborns and are the most common disorders among inherited metabolic disorders [8]. However, there are few published reports regarding the relationship between MRC disorders and SUDI. Studies of MRC disorders have not progressed because of technical difficulties or variability in clinical manifestations [9]. In sudden death cases especially, clinical features are unclear and postmortem changes complicate molecular analysis.

In the present study, we performed metabolic autopsy in 13 Japanese cases of SUDI in order to determine whether MRC disorders could be detected or not. We performed fat staining of liver and postmortem

Abbreviations: CS, citrate synthetase; FAODs, fatty acid oxidation disorders; MRC, mitochondrial respiratory chain; OXPHOS, oxidative phosphorylation; SIDS, sudden infant death syndrome; SUDI, sudden unexpected death in infancy.

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acylcarnitine analysis according to the previous methods. In addition, we analyzed MRC enzyme activity in frozen organs as well as in postmortem cultured fibroblasts. With such metabolic autopsy, we were able to detect one probable case and one possible case of MRC disorders. These cases might not have been identified without metabolic autopsy. MRC disorders are important diseases causing SUDI and metabolic autopsy might be helpful for forensic scientists and pediatricians to diagnose MRC disorders that might not otherwise be identified.

2. Materials and methods

2.1. Subjects

Between October 2009 and September 2011, forensic autopsy was performed on 588 cases at our institute, 22 of whom were under 12 months of age. Following macroscopic examination, nine cases could be diagnosed but 13 cases (Table 1) did not have any characteristic appearance and remained undiagnosed. In this study, we reviewed these 13 undiagnosed cases (8 males, 5 females) with age ranging from 1 to 10 months.

2.2. Autopsy

Autopsies were performed within 24 h following death. Blood was obtained from the femoral vein. Heart and liver specimens were immediately cut and frozen at −80 °C. Dermis, which was cut and sterilized, was cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum, 1% penicillin streptomycin glutamine, and 2.5% amphotericin B (Life Technologies, Indianapolis, IN). Once cultures were established, fibroblasts were frozen at −80 °C.

2.3. Sudan III staining

Liver samples preserved in 4% phosphate-buffered formaldehyde solution were frozen, cut into 10-μm sections, and stained by the Sudan III method for fat staining.

2.4. Postmortem blood acylcarnitine analysis by tandem mass spectrometry

Whole blood samples obtained at autopsy were blotted onto one spot on Guthrie cards. They were subjected to acylcarnitine analysis by tandem mass spectrometry and compared with the previously determined normal range [6].

Table 1
SUDI cases.

Case no.	Age/sex	Height/weight (cm/kg)	Circumstances	Fever	Remarks
1	4 mo/M	68/7.5	Sleeping	−	
2	10 mo/F	70/8.8	Sleeping	−	Sister: undiagnosed encephalitis
3	10 mo/F	71/7.7	Sleeping	+	Cesarean section
4	9 mo/M	67/7.5	Sleeping	−	
5	4 mo/M	60/5.7	Sleeping	−	Hydrocephalia
6	6 mo/M	68/8.0	Sleeping	−	
7	1 mo/F	51/3.6	Sleeping	−	Twins, preterm birth
8	10 mo/M	72/9.9	Sleeping	−	Developmental disease (right side of the body paralysis)
9	6 mo/F	64/8.9	Sleeping	−	Bronchitis
10	4 mo/M	65/7.4	Sleeping	−	Cesarean section
11	1 mo/M	58/4.8	Sleeping	−	
12	5 mo/M	59/4.2	Sleeping	−	Preterm birth
13	2 mo/F	53/3.9	Sleeping	−	Low-birth-weight infant

Abbreviations: F, female; M, male; mo, month; SUDI, sudden unexpected death in infancy.

2.5. Enzyme analysis

The activity of mitochondrial respiratory chain complexes I, II, III, and IV was assayed in the crude post-600-g supernatant of heart and liver, and in isolated mitochondria from skin fibroblasts as described previously [10]. The activity of each complex was presented as a percent ratio relative to the mean value [9]. The activity of complexes I, II, III, and IV was also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker or complex II activity [10].

2.6. Ethics

This study was approved by the Ethics Committee of the Osaka University Graduate School of Medicine.

3. Results

3.1. Microscopic examination

One of the common features in diagnosing MRC disorders is hepatic steatosis. We therefore performed Sudan III staining to examine whether vacuoles caused by fatty degeneration were present in hepatocytes. Diffuse microvesicular steatosis was detected in case 5 (Fig. 1A). No Sudan III-positive vacuole was detected in case 13 (Fig. 1B) and the other cases, for example, case 2 (Fig. 1C).

3.2. Postmortem blood acylcarnitine analysis

We performed acylcarnitine analysis by tandem mass spectrometry using whole blood samples. In all samples, data were within the normal range. These data suggested that no case was affected by FAODs (data not shown).

3.3. Enzyme analysis of MRC complexes in heart, liver, and cultured fibroblasts

The enzyme activity of each complex was compared with the CS ratio and complex II ratio. Lower than 20% activity of any complex in a tissue or lower than 30% activity of any complex in a cell line meets the major criteria. Lower than 30% activity of any complex in a tissue or lower than 40% activity of any complex in a cell line meets the minor criteria according to Bernier et al. [11].

In heart, 11 cases of complex I activity met the major criteria of MRC disorders and one case of complex I activity met the minor criteria (Fig. 2A). In liver, three cases of complex I activity met the major criteria of MRC disorders and four cases of complex I activity met the minor criteria (Fig. 2B). In cultured fibroblasts, one case (case 5) of complex I activity met the major criteria of MRC disorders and one case (case 13) of complex I activity met the minor criteria (Fig. 2C, Table 2). The activity of complexes II, III, and IV was maintained in almost all cases.

3.4. Diagnosis

A definite diagnosis is defined as the identification of either two major criteria or one major plus two minor criteria. A probable diagnosis is defined as either one major plus one minor criterion or at least three minor criteria. A possible diagnosis is defined as either a single major criterion or two minor criteria, one of which must be clinical [11].

All the cases had a clinical symptom of sudden death, meeting one minor criterion. In the enzyme activity, eleven cases (cases 2, 4–13) met the major criteria and we could make a probable diagnosis in these 11 cases. The other two cases (cases 1 and 3) met the minor criteria and we could make a possible diagnosis.

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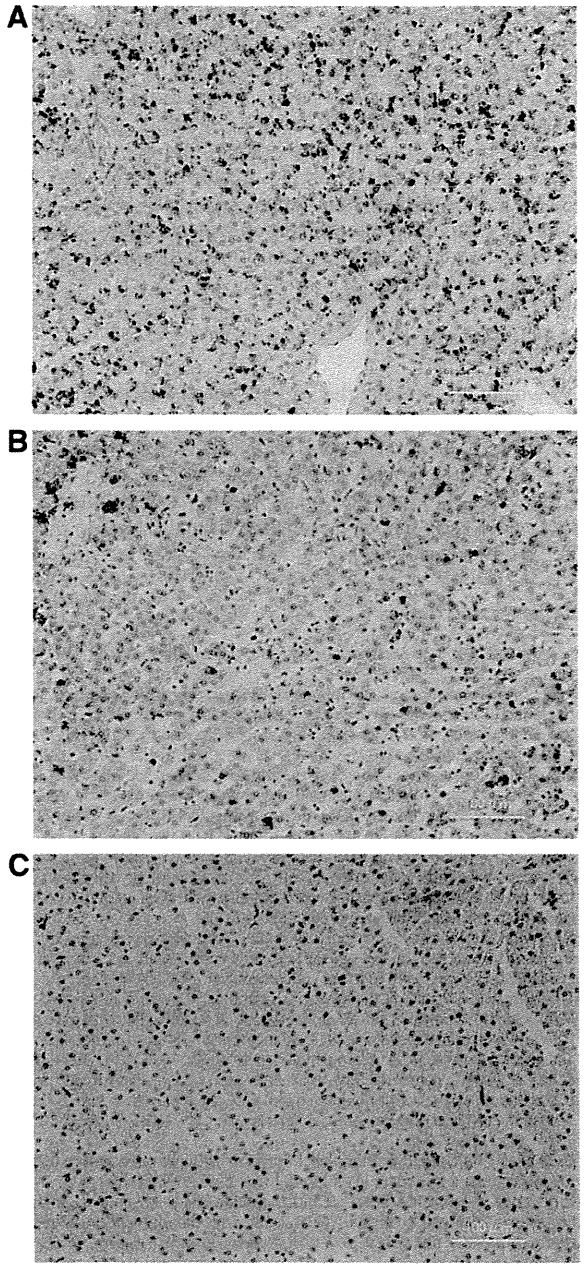

Fig. 1. Microscopic examination of liver (Sudan III staining): (A) case 5, (B) case 13, and (C) case 2. Diffuse microvesicular steatosis was detected in case 5 (A). No Sudan III-positive vacuole was detected in case 13 (B) and the other cases, for example, case 2 (C).

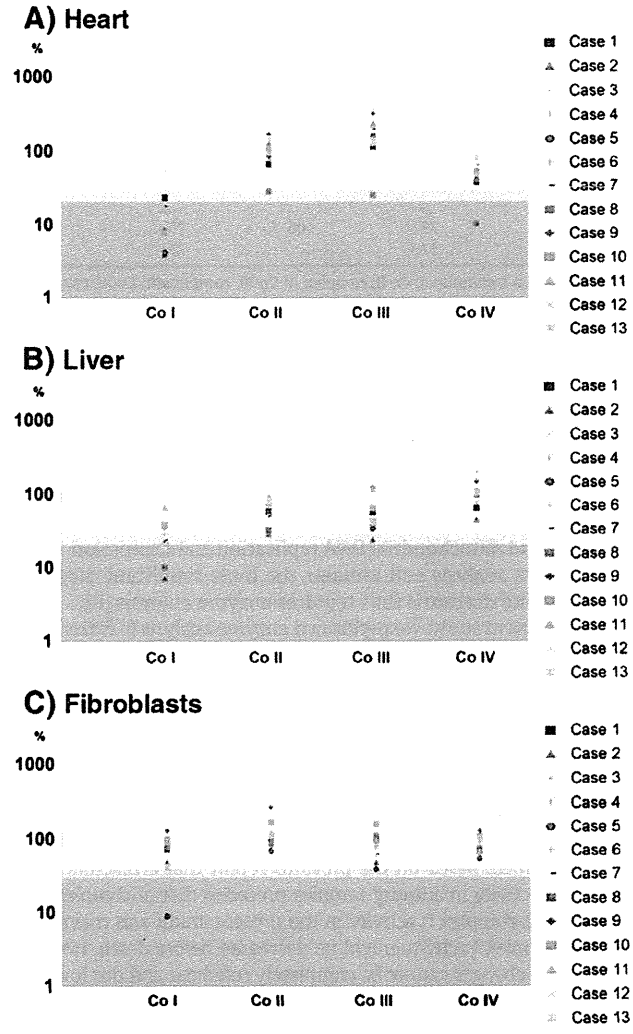

Fig. 2. Enzyme activity of MRC complexes in heart (A), liver (B), and cultured fibroblasts (C). In heart, 11 cases of complex I activity were under 20% of the CS ratio, meeting the major criteria and one case of complex I activity was under 30% of the CS ratio, meeting the minor criteria (A). In liver, three cases of complex I activity were under 20% of the CS ratio, meeting the major criteria and four cases of complex I activity were under 30% of the CS ratio, meeting the minor criteria (B). In cultured fibroblasts, one case (case 5) of complex I activity was under 30% of the CS ratio, meeting the major criteria and one case (case 13) of complex I activity was under 40% of the CS ratio, meeting the minor criteria (C). The activity of complexes II, III, and IV was maintained in almost all cases. The enzyme activity of each complex was compared with the CS ratio. Lower than 20% activity in a tissue or lower than 30% activity in a cell line (dark blue) meets the major criteria. Lower than 30% activity in a tissue or lower than 40% activity in a cell line (light blue) meets the minor criteria. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Mitochondria are essential organelles that exist in all nucleated mammalian cells. They provide the energy required for normal cell function through oxidative phosphorylation (OXPHOS). OXPHOS includes MRC complexes (complexes I, II, III, and IV) and ATP synthase (complex V) [12], which use reduced coenzymes from the tricarboxylic acid cycle and molecular oxygen, generating cellular energy in the form of ATP [13].

The infantile or early neonatal period demands high energy. Patients with MRC disorders are unable to produce adequate energy, which may thus compromise them in the first days of life or during infancy. MRC disorders affect most organ systems and present variable clinical manifestations from prenatal complications through acute neonatal decompensation and death to adult-onset disorders. Therefore, it is not surprising that MRC disorders are also one of the causes of SUDI. However, there are few reports on a relationship between MRC disorders and SUDI [12,14].

We have previously reviewed SUDI cases with respect to FAODs and found a case of carnitine palmitoyltransferase II deficiency [6]. In that study, we advocated the importance of metabolic autopsy [15], including fat staining of liver, postmortem acylcarnitine analysis, and genetic analysis. Using this protocol, most FAODs, some amino acid oxidation disorders, and some organic acid oxidation disorders could be diagnosed.

However, MRC disorders are difficult to diagnose. First, they present variable clinical manifestations and non-specific features such as failure to thrive or hepatic, cardiac, renal, gastrointestinal, endocrine, hematological, or other symptoms [10,16]. Second, although blood

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Table 2
Enzyme assay of mitochondrial respiratory chain complexes in cultured fibroblasts.

	Enzyme activity (%)[a]			
	Co I	Co II	Co III	Co IV
Case 5				
CS ratio	9	66	38	53
Co II ratio	13	–	71	58
Case 13				
CS ratio	39	106	76	98
Co II ratio	37	–	73	92

Abbreviations: Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthetase.
[a] Relative to mean CS and Co II of the normal controls.

lactate levels and muscle morphology can be used as a screening test, some confirmed patients were normal [10]. Third, genomic mutational analysis is difficult because MRC complexes are composed of 13 subunits encoded by mitochondrial DNA and over 70 subunits encoded by nuclear genes. In addition, nuclear genes are related to many assembly factors, membrane dynamics, nucleotide transport synthesis, and mitochondrial DNA replication and expression. Therefore, enzyme analysis still remains the most significant diagnostic tool. A definite diagnosis thus requires enzyme analysis [8].

In the present study, we performed enzyme analysis in frozen heart, frozen liver, and cultured fibroblasts. Eleven cases were supposed to be a probable diagnosis and two cases were supposed to be a possible diagnosis. However, it seemed unlikely that such a high proportion would have real MRC disorders. Did we have to take the effect of postmortem changes into consideration?

For forensic autopsy, organ specimens are often preserved in formaldehyde solution and sometimes frozen. These specimens are susceptible to postmortem changes and, therefore, correct enzyme analysis is hard to be performed. Based on the previous report that artifactual loss of complex II activity in autopsy samples preceded that of complex I and the data that complex II activity in the present study was maintained, this low complex I activity might be decreased before death. However, postmortem changes cannot be completely ruled out and this low complex I activity may not therefore be consistent with premortem activity.

We therefore analyzed activity in cultured fibroblasts. Cultured fibroblasts are not affected by postmortem changes and, therefore, reflect premortem information more accurately. In cultured fibroblasts, one case (case 5) of complex I activity met the major criteria and one case (case 13) of complex I activity met the minor criteria. In case 5, complex I activity was distinctively decreased. Sudan III staining of the case revealed hepatic steatosis, consistent with Reye-like syndrome. Reye-like syndrome is one of the characteristic features of MRC disorders [9]. We could therefore make a probable diagnosis (case 5) and a possible diagnosis (case 13) from metabolic autopsy with postmortem cultured fibroblasts.

Case 5 had hydrocephalia and case 13 was a low-birth-weight infant. However, neither was severe. Macroscopic examination did not reveal any abnormal appearance and microscopic examination showed no pathological findings except for steatosis. These cases might not have been identified without postmortem cultured fibroblasts. As with such cases, some MRC disorders reveal no clinical manifestation and no pathological characteristic. We believe it is important to perform metabolic autopsy with postmortem cultured fibroblasts when encountering SUDI cases.

We emphasized the advantage of metabolic autopsy with cultured fibroblasts. First, despite lacking obvious preceding symptoms, MRC disorders could be diagnosed. Second, cultured cells are the only method to retrieve premortem information from the deceased. Third, even frozen samples are affected by postmortem changes and may lead to a false positive diagnosis. However, we have to discuss the disadvantage. MRC disorders showed tissue specificity and the activity of cultured fibroblasts represent normal in some cases. Some of the low complex I activity in heart or liver could represent premortem MRC disorders despite normal activity in cultured fibroblasts. Thus, other molecular investigations may well be added to enzyme analysis. Recently, systematic gene analysis using next-generation sequencing has been reported for the diagnosis of patients with MRC disorders [17]. Further investigations are thus needed.

In conclusion, we detected one probable case and one possible case of MRC disorders among 13 Japanese cases of SUDI. MRC disorders are one of the important inherited metabolic disorders causing SUDI. We advocate metabolic autopsy with postmortem cultured fibroblasts in SUDI cases.

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