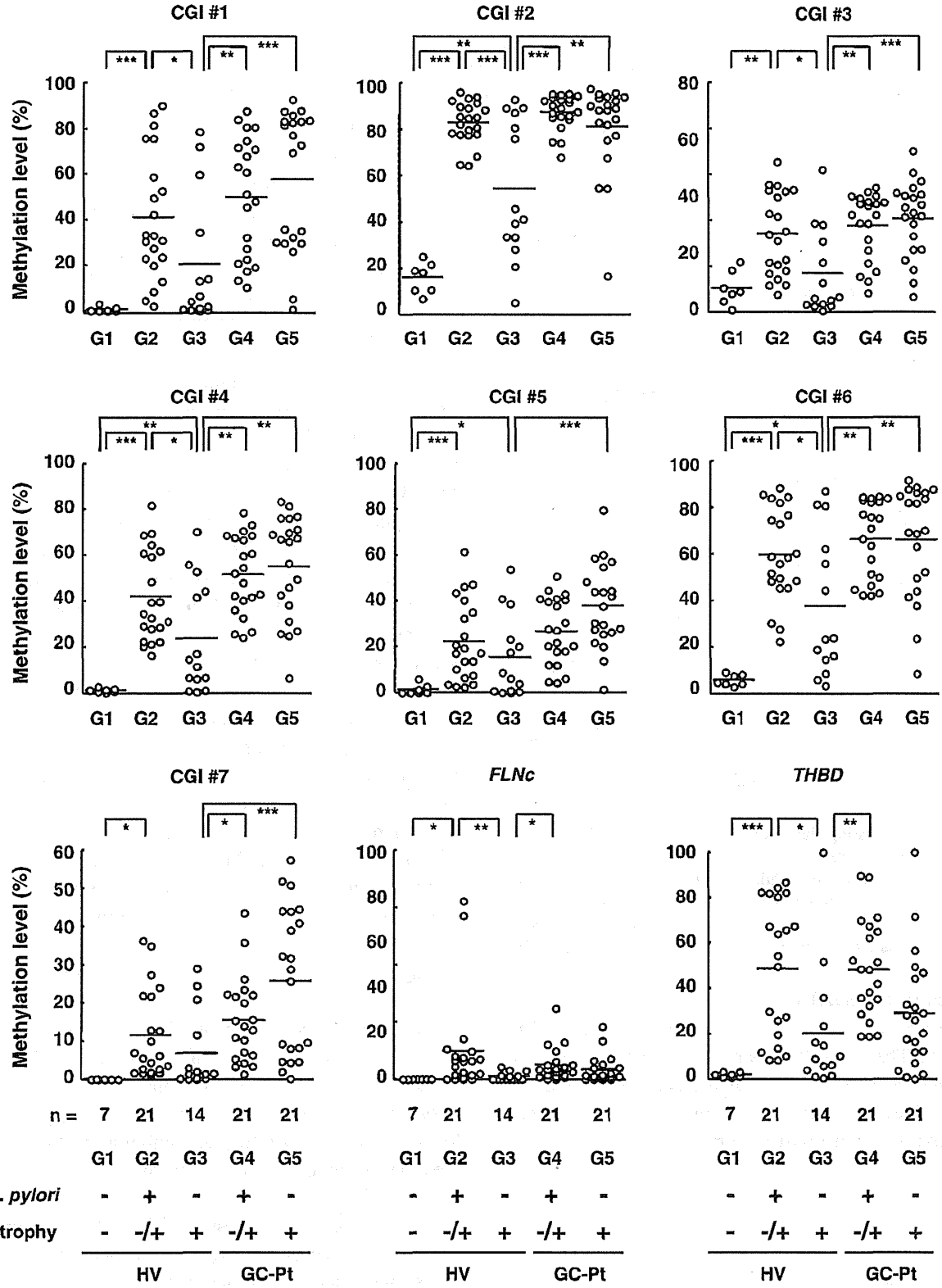

Fig. 2 Methylation levels of the seven CGIs and two currently available markers, *FLNc* and *THBD*, in the validation set. The *horizontal line* represents the mean methylation level in each group. Methylation levels of the seven CGIs in Group 5 (G5) were significantly higher than those in G3 ($P < 0.01$), but there were no significant differences for the two currently available markers. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Table 2 AUC and OR for new and currently available markers

CGI no.	Gene symbol	AUC	95% CI	*P* value	OR	95% CI	*P* value
#1	*EMX1*	0.84	0.70–0.97	<0.001	23.8	3.7–153	<0.001
#2	*miR663*	0.78	0.62–0.94	0.006	26.7	2.8–258	0.005
#3	*NKX6-1*	0.84	0.69–0.99	<0.001	15.0	2.8–80.1	0.002
#4	*OTP*	0.83	0.69–0.97	0.001	36.0	3.7–354	0.002
#5	*OPLAH*	0.83	0.69–0.98	0.001	15.6	2.9–83.5	0.001
#6	*CYP1B1*	0.78	0.62–0.94	0.006	12.7	2.1–76.7	0.006
#7	*NEFM*	0.84	0.71–0.98	<0.001	23.8	3.7–153	<0.001
–	*FLNc*	0.69	0.51–0.87	0.055	5.7	1.2–25.9	0.025
–	*THBD*	0.65	0.45–0.84	0.152	5.0	1.1–21.8	0.032

CGI CpG island, *AUC* area under the curve, *CI* confidence interval, *OR* odds ratio

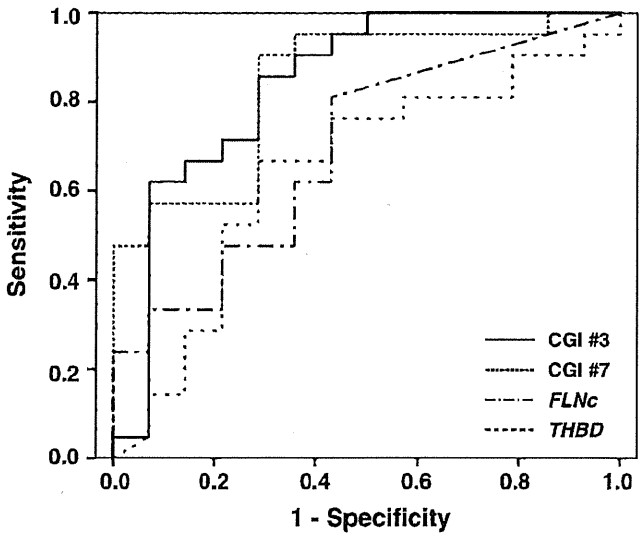

Fig. 3 Receiver-operating characteristic (ROC) curves of CGI #3 and #7, whose AUC values were the largest in the seven CGIs, are shown with those of two currently available markers, *FLNc* and *THBD*. *Black line*, *dotted line*, *dot-and-dash line*, and *dashed line* show ROC curves of CGI #3, #7, *FLNc*, and *THBD*, respectively. The AUC values of CGI #3 and #7 were larger than those of *FLNc* and *THBD*

the two currently available markers, *FLNc* and *THBD*, were 5.7 (95% CI 1.2–25.9) and 5.0 (95% CI 1.1–21.8), respectively. These results clearly showed that the methylation levels of the seven CGIs had greater power than the two currently available markers to estimate gastric cancer risk in individuals with past infection.

Discussion

In the present study, by carrying out genome-wide methylation analysis of gastric cancer patients (GC-Pt) and healthy volunteers (HV), both with past infection, we screened seven gastric cancer risk markers that are highly informative in individuals with past infection. Their usefulness was validated in 35 individuals (21 GC-Pt and 14 age-matched HV). To our knowledge, this is the first study that has evaluated epigenetic gastric cancer risk markers in individuals with past infection, and these markers are expected to be especially useful. This is because the number of individuals with past infection is increasing as more and more people receive *H. pylori* eradication therapy [18], but the usefulness of the current methods for gastric cancer risk estimation, i.e., a combination of the detection of *H. pylori* infection and the serum pepsinogen test, in this population has not been established [18–20].

None of the seven CGIs were located in promoter regions. We analyzed the association between the methylation levels of the seven CGIs and the expression levels of genes close to them, but no association was observed for any of the seven CGIs (data not shown). This was in line with the current knowledge that DNA methylation of only promoter CGIs consistently causes gene silencing, but that methylation of gene bodies may or may not be associated with increased expression [14, 21, 22]. The lack of association between methylation and gene expression supported the hypothesis that the methylation of these seven CGIs reflects the degree of overall epigenomic damage in gastric stem cells, and that the degree of epigenomic damage, and not the change of expression of individual genes, is associated with gastric cancer risk.

Epigenomic damage induced by *H. pylori* infection is one of the major causes of gastric cancer [23–26], but it is not known whether the epigenomic damage is independent of other risk factors. For example, salt intake is a risk factor for gastric cancer [27, 28], and although it does not induce methylation in gastric mucosae by itself in a Mongolian gerbil model [29, 30], it shows synergistic effects with *H. pylori* on cancer development [31]. It is not known yet whether epigenomic damage in the gastric mucosa provides independent information from past salt exposure or whether the exposure is already reflected in methylation levels. Multivariate analysis in a large cohort with a reliable record of history of salt intake will clarify this issue, and might provide a risk marker that complements the epigenetic gastric cancer risk markers.

In conclusion, we identified seven CGIs whose methylation levels are increased after *H. pylori* infection, and

are associated with gastric cancer risk even in individuals with past infection. These seven CGIs are promising candidate markers to estimate gastric cancer risk.

Acknowledgments This study was supported by Grants-in-Aid for Pioneering Basic Research and for the Third-term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour and Welfare, Japan.

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Cancer Letters 330 (2013) 33–40

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Comprehensive DNA methylation and extensive mutation analyses reveal an association between the CpG island methylator phenotype and oncogenic mutations in gastric cancers

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ARTICLE INFO

Article history:
Received 10 October 2012
Received in revised form 12 November 2012
Accepted 12 November 2012

Keywords:
Epigenetics
Aberrant DNA methylation
CIMP
Mutation
Gastric cancer

ABSTRACT

Recent development of personal sequencers for extensive mutation analysis and bead array technology for comprehensive DNA methylation analysis have made it possible to obtain integrated pictures of genetic and epigenetic alterations on the same set of cancer samples. Here, we aimed to establish such pictures of gastric cancers (GCs). Comprehensive methylation analysis of 30 GCs revealed that the number of aberrantly methylated genes was highly variable among individual GCs. Extensive mutation analysis of 55 known cancer-related genes revealed that 19 of the 30 GCs had 24 somatic mutations of eight different genes (*CDH1*, *CTNNB1*, *ERBB2*, *KRAS*, *MLH1*, *PIK3CA*, *SMARCB1*, and *TP53*). Integration of information on the genetic and epigenetic alterations revealed that the GCs with the CpG island methylator phenotype (CIMP) tended to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*. This is one of the first studies in which both genetic and epigenetic alterations were extensively analyzed in the same set of samples. It was also demonstrated for the first time in GCs that the CIMP was associated with oncogene mutations.

1. Introduction

Both genetic and epigenetic alterations are important for human carcinogenesis [1,2]. Genetic alterations are responsible for activation of oncogenes and inactivation of tumor-suppressor genes [2]. In human gastric cancers (GCs), oncogenes activated by mutations include *CTNNB1* (β-catenin), *ERBB2*, and *PIK3CA* [3–10], and tumor-suppressor genes inactivated by mutations include *CDH1* (E-cadherin), *CDKN2A* (*p16*), *TP53*, and *ARID1A* [11,12]. Even by whole exome sequencing of GCs, the vast majority of driver genes identified were known cancer-related genes, and novel genes identified, such as *ARID1A* and *FAT4*, had only low incidences of mutations [11,12]. This indicates that extensive mutation analysis of a large number of known cancer-related genes can provide an overall picture of a cancer sample, and this is now possible with high speed and low cost by using next-generation personal sequencers [13,14].

Epigenetic alterations, namely aberrant DNA methylation of promoter CpG islands (CGIs), are also responsible for inactivation of various tumor-suppressor genes [1]. DNA methylation statuses of the entire genome can be now comprehensively analyzed using microarray technologies, and bead array technology is especially useful for its quantitative measurement [15]. In GCs, tumor-suppressor genes inactivated by promoter methylation include *CDH1*, *CDKN2A*, *FHL1*, *LOX*, *MLH1*, and *SFRP* family genes (*SFRP1*, *SFRP2*, and *SFRP5*) [16–21]. These tumor-suppressor genes are more frequently inactivated by aberrant methylation than by genetic alterations in GCs [22]. In addition, aberrant methylation is induced in gastric mucosae by *Helicobacter pylori* (*H. pylori*)

Abbreviations: GC, gastric cancer; CGI, CpG island; *H. pylori*, *Helicobacter pylori*; CIMP, CpG island methylator phenotype; EB virus, Epstein–Barr virus; TSS, transcription start site; COSMIC, Catalogue Of Somatic Mutations In Cancer.

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http://dx.doi.org/10.1016/j.canlet.2012.11.022

infection [23,24], a well-established major inducer of human GCs [25]. The frequent inactivation of tumor-suppressor genes by aberrant methylation and the deep involvement of *H. pylori* infection in its induction indicate the importance of epigenetic alterations in GCs.

Not only in GCs but also in other types of cancers, a subgroup of cancers is known to have frequent aberrant DNA methylation of CGIs, referred to as the CpG island methylator phenotype (CIMP). The CIMP was first described in colorectal cancers [26], and is associated with unique clinicopathological features. For example, the CIMP is associated with poor prognosis in colorectal cancers, lung cancers, and neuroblastomas [27–29]. In contrast, depending on the number and set of genes used for the determination of the CIMP status, the CIMP can be associated with either poor or good prognosis in GCs [30–33]. The CIMP in specific cancers is associated with genetic alterations, such as mutations of *BRAF*, *KRAS*, and *PIK3CA* in colorectal cancers [34–37], and amplification of *ERBB2* in breast cancers [38]. In contrast, little is known on a specific association between the CIMP and genetic alterations in GCs.

In this study, we aimed to establish integrated pictures of genetic and epigenetic alterations of GCs. To this end, we conducted comprehensive analysis of DNA methylation statuses using bead array technology, and extensive analysis of mutations of 55 known cancer-related genes using a next-generation personal sequencer.

2. Materials and methods

2.1. Samples

Thirty GC samples were obtained from patients who underwent gastrectomy with informed consents. Three normal gastric mucosae samples were obtained endoscopically from healthy volunteers without *H. pylori* infection with informed consents. The study was approved by the Institutional Review Boards. The samples were stored in RNAlater (Life Technologies, Carlsbad, CA) at −80 °C until the extraction of genomic DNA (GC samples and normal gastric mucosae samples) and RNA (normal gastric mucosae samples). Clinical information of the 30 GCs is shown in Supplementary Table 1. The status of Epstein–Barr (EB) virus infection was evaluated by PCR using primers specific to genomic DNA of EB virus (forward, CGGTATTATGTTTTTGGTATGTGTA; reverse, ATAACAACAACGTCATAAAAACCAC), and no infection was present in the 30 GCs.

Genomic DNA was extracted from GC and normal gastric mucosae samples by the phenol/chloroform method, and was quantified by using a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan).

2.2. Analysis of DNA methylation

Analysis of DNA methylation was performed using an Infinium HumanMethylation450 BeadChip array, which covered 482,421 CpG sites (Illumina, San Diego, CA) as described previously [39]. CpG sites with low signals (signal <500, 0.19–2.19% of total CpG sites) were excluded from further analyses. The methylation level of each CpG site was represented by β values which ranged from 0 (unmethylated) to 1 (fully methylated).

A total of 193,531 genomic "segments" were defined by their location against a transcription start site (TSS) [TSS1500 (regions between 200 bp upstream and 1500 bp upstream from TSS), TSS200 (200 bp upstream region from TSS), 5′-UTR, 1st exon, gene body, 3′-UTR, and intergenic regions] and their relative location against a CGI (N Shelf, N Shore, CGI, S Shore, S Shelf, and non-CGI). A genomic segment >500 bp was further divided into genomic "blocks". A genomic block was defined as a 500-bp region from an initial CpG site (probe), and the next genomic block started from the next CpG site (Supplementary Fig. 1). A genomic segment ⩽500 bp was counted as one genomic block. A total of 282,805 genomic blocks were produced, and 276,456 genomic blocks on autosomes were analyzed to enable comparison between males and females. A DNA methylation level of a genomic block was evaluated using the average of β value of the CpG sites within the block. A genomic block was considered as methylated when its β value was 0.4 or more, and as unmethylated when its β value was 0.2 or less.

2.3. Analysis of sequence variations

A library DNA containing 226 amplicons of 55 cancer-related genes was prepared from a sample by multiplex PCR using 50 ng of genomic DNA and an Ion *AmpliSeq* Cancer Panel Kit (Life Technologies) with 36 customized primers (Supplementary Table 2). The 226 amplicons covered the vast majority of samples with mutations reported (91.9% or more) for 15 oncogenes and the *TP53* tumor-suppressor gene (83.1%), and variable fractions of samples with mutations reported (3.3–88.5%) for 39 genes (Supplementary Table 3). Then, the entire library DNA was uniquely barcoded by using an Ion Xpress Barcode Adaptors 1-16 Kit (Life Technologies). The barcoded libraries from five to six samples were pooled, and mixed with Ion Spheres for emulsion PCR using the Ion OneTouch System (Life Technologies) with an Ion OneTouch Template Kit (Life Technologies). From the product of emulsion PCR, the complexes of Ion Spheres with amplified DNA were enriched by using Ion OneTouch ES (Life Technologies) and were loaded onto an Ion 316 chip (Life Technologies). Sequencing was performed by using Ion PGM Sequencer (Life Technologies) with an Ion Sequencing Kit (Life Technologies). Obtained sequences were mapped onto the human reference genome hg19, and sequence variations with frequencies of 10% or more were identified by using CLC Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark). Common SNPs were excluded from further analysis. Reading depths of individual regions analyzed are shown in Supplementary Table 4.

2.4. Dideoxy sequencing

A region containing a sequence variation identified was amplified using 20 ng of genomic DNA with primers listed in Supplementary Table 5. The PCR product was purified by a DNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA), and directly cycle-sequenced by using a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare, Buckinghamshire, UK) and an ABI PRISM 310 automated DNA sequencer (PE Biosystems).

2.5. Analysis of gene expression by GeneChip oligonucleotide microarray

Gene expression levels in normal gastric mucosae were analyzed by using the GeneChip Human Genome U133 Plus 2.0 microarray (Affymetrix, Santa Clara, CA) as described [40]. Genes with signal intensities of 250 or more were defined as expressed genes.

2.6. Cluster analysis

Unsupervised hierarchical clustering analysis was performed by using R 2.15 [R Core Team (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/] with the Heatplus package [Alexander Ploner (2011) Heatplus: Heatmaps with row and/or column covariates and colored clusters, R package version 2.2.0.] from Bioconductor [41]. The Euclidean distance was used as distance function both for samples and genes. Due to the limitation in the calculation algorithm for the hierarchical clustering, 25,000 elements or less were analyzed.

2.7. Survival curve

Survival curves were analyzed using the Kaplan-Meier method, and the Kaplan-Meier curve was drawn by using SPSS 13.0J (SPSS, Chicago, IL, USA).

2.8. Statistical analysis

The association between the CIMP and oncogene mutations, and that between genes aberrantly methylated in GCs and target genes of polycomb repressive complex (PRC) 2 in human embryonic stem (ES) cells were tested by the chi-square test. The differences in the survival rates among groups were evaluated using the Mantel-Cox test.

3. Results

3.1. Comprehensive analysis of DNA methylation profiles

DNA methylation levels were compared between GCs and normal gastric mucosae. First, using all the 276,456 genomic blocks, some GCs, such as S24TP, S33TP, and S37TP, had a larger fraction of aberrantly methylated blocks than other GCs, such as S2TP, S4TP, and S15TP (Fig. 1 and Supplementary Fig. 2). Second, the analysis was conducted using 6877 TSS200 CGIs unmethylated in normal gastric mucosae (genes unmethylated in normal gastric mucosae) because a TSS200 CGI is known to play a critical role in methylation-silencing [42]. The number of aberrantly methylated genes ranged from three to 1211. Third, we focused on TSS200 CGIs of genes with positive expression in normal cells but aberrantly methylated in cancer cells because this group of genes is known to frequently contain driver genes in carcinogenesis [43]. Using 263 TSS200 CGIs whose downstream genes were expressed in

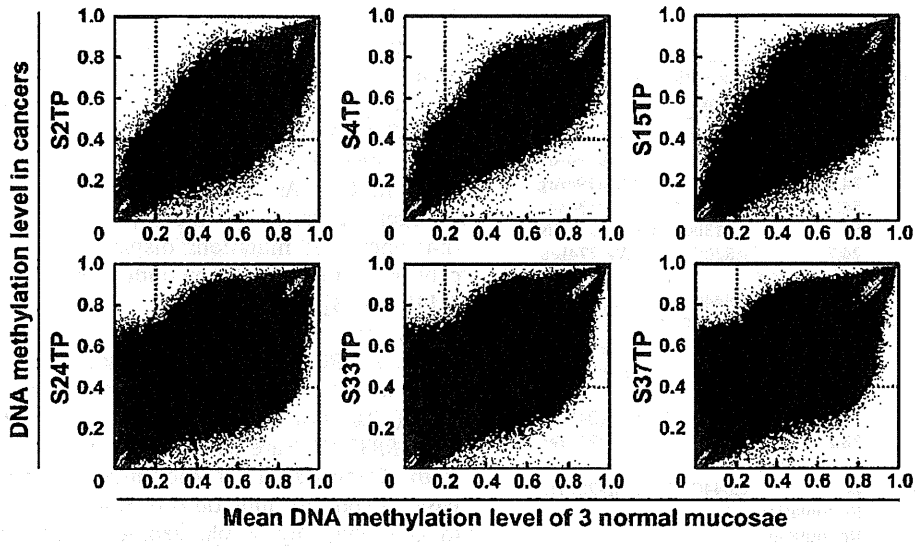

Fig. 1. Comprehensive analysis of DNA methylation profiles in GCs. DNA methylation levels were compared between GCs and normal gastric mucosae for the 276,456 genomic blocks. S24TP, S33TP, and S37TP (lower three panels) had a larger fraction of aberrantly methylated genes (yellow-colored areas) than S2TP, S4TP, and S15TP (upper three panels). The vertical and horizontal axes indicate the methylation levels in GCs and the mean methylation levels of three normal mucosae, respectively.

normal gastric mucosae and aberrantly methylated in one or more GCs (methylation-silenced genes), the number ranged from 0 to 166. These results showed that the number of aberrantly methylated genes was highly variable among individual GCs.

3.2. Extensive mutation analysis of the 55 cancer-related genes

Mutations were analyzed for the 55 cancer-related genes. Among the 30 GCs, 22 GCs had 30 sequence variations of at least one gene (Table 1 and Supplementary Table 6), and all the 30 sequence variations were confirmed by dideoxy sequencing (Supplementary Fig. 3). The confirmed sequence variations were analyzed whether or not they were somatic mutations using corresponding non-cancerous tissues. The 24 of the 30 sequence variations were shown to be somatic mutations (Fig. 2 and Table 1), and were present in 19 GCs. Among the 24 mutations, 22 were missense mutations, and two were nonsense mutations. Three GCs (S5TP, S13TP, and S33TP) had two or more mutations of different genes. Four oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*, and four tumor-suppressor genes, *CDH1*, *MLH1*, *SMARCB1*, and *TP53*, were mutated. *TP53* was most frequently mutated (43%, 13 of the 30 GCs), and *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA* were mutated in two GCs. These results showed that 63% of GCs (19 out of the 30 GCs) had at least one somatic mutation of known cancer-related genes.

3.3. The association between the CIMP and mutations of oncogenes

Unsupervised hierarchical clustering analysis was conducted first using DNA methylation profiles of 25,000 genomic blocks randomly selected from all the 276,456 genomic blocks. However, the numbers of aberrantly methylated genes in GCs of different clusters did not appear to be different (Supplementary Fig. 4). Then, we again conducted unsupervised hierarchical clustering using DNA methylation profiles of CGIs, namely 25,000 genomic blocks randomly selected from 59,992 blocks with CGIs (Fig. 3A). This time, clusters I ($n = 3$) and IIb ($n = 13$) contained GCs with a larger number of aberrantly methylated genes than GCs in cluster IIa ($n = 14$). Among the 16 GCs in clusters I and IIb, seven GCs were shown to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*.

Thirdly, using DNA methylation profiles of 6877 genes unmethylated in normal gastric mucosae, two major clusters were observed (Fig. 3B). Cluster III ($n = 11$) contained GCs with a relatively large number of aberrantly methylated genes, and seven of the 11 GCs of this cluster were shown to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*. In contrast, cluster IV ($n = 19$) contained GCs with a relatively small number of aberrantly methylated genes, and none of the 19 GCs in this cluster had mutations of oncogenes. The difference was markedly statistically significant ($P = 7.15 \times 10^{-5}$), and GCs in cluster III and IV were considered to be the CIMP-positive [CIMP(+)] and the CIMP-negative [CIMP(−)], respectively.

Fourth, using DNA methylation profiles of the 263 methylation-silenced genes, three major clusters were produced (Fig. 3C). Cluster V ($n = 3$) contained GCs with the largest number of aberrantly methylated genes, and two of the three GCs were shown to have mutations of *PIK3CA*. Cluster VIa ($n = 8$) contained GCs with a relatively larger number of aberrantly methylated genes than GCs in cluster VIb ($n = 19$). Five of the eight GCs in this cluster were shown to have mutations of oncogenes, *CTNNB1*, *ERBB2*, *KRAS*. Clusters VIb contained the same sets of GCs as cluster IV, the previous clustering, except for one. These results showed that the CIMP(+) GCs were associated with mutations of oncogenes, such as *CTNNB1*, *ERBB2*, *KRAS* and *PIK3CA*, in GCs.

3.4. Possible association between the CIMP and good prognosis

To analyze an association between the CIMP status and prognosis of patients, Kaplan-Meier curves were drawn using overall survival (OS). Using the CIMP status based on the DNA methylation of the 6877 genes unmethylated in normal gastric mucosae, it was revealed that the prognosis of the CIMP(+) patients (Cluster III in Fig. 3B) tended to be better than that of the CIMP(−) patients (Cluster IV in Fig. 3B) ($P = 0.285$; Fig. 4). Also, using the CIMP status based on the methylation of the 263 methylation-silenced genes, the prognosis of the CIMP(+) patients (Cluster V and VIa in Fig. 3C) tended to be better than that of the CIMP(−) patients (Cluster VIb in Fig. 3C) ($P = 0.285$; Supplementary Fig. 5). These results suggested that the CIMP(+) status is possibly associated with good prognosis in GCs.

Table 1
List of somatic mutations identified in the 30 GCs.

Sample #	Sample name	Gene	Coverage	Variant frequencies	Nucleotide change	Amino acid change
1	S1TP	*CDH1*	339	10.3	c.1198G > A	p.Asp400Asn
2	S2TP	*TP53*	496	34.1	c.581T > G	p.Leu194Arg
3	S4TP	*TP53*	438	74.2	c.581T > G	p.Leu194Arg
4	S5TP	*KRAS*	1626	54.4	c.38G > A	p.Gly13Asp
		SMARCB1	50	56	c.1130G > A	p.Arg377His
5	S6TP	*TP53*	2077	24.7	c.820G > C	p.Val274Leu
6	S9TP			No mutation		
7	S11TP	*TP53*	10,211	53.4	c.844C > T	p.Arg282Trp
8	S12TP	*ERBB2*	24,516	63.8	c.2264T > C	p.Leu755Ser
9	S13TP	*TP53*	70	15.7	c.478A > G	p.Met160Val
		ERBB2	482	23.9	c.2264T > C	p.Leu755Ser
10	S14TP			No mutation		
11	S15TP	*TP53*	534	40.3	c.743G > A	p.Arg248Gln
12	S16TP	*TP53*	453	36.2	c.660T > G	p.Tyr220Ter
13	S17TP			No mutation		
14	S18TP	*TP53*	1946	26.5	c.844C > T	p.Arg282Trp
15	S19TP			No mutation		
16	S20TP			No mutation		
17	S22TP			No mutation		
18	S23TP	*TP53*	565	67.8	c.537T > A	p.His179Gln
19	S24TP			No mutation		
20	S32TP			No mutation		
21	S33TP	*MLH1*	4092	45.4	c.1744C > G	p.Leu582Val
		CTNNB1	11,994	20.5	c.101G > A	p.Gly34Glu
		PIK3CA	276	49.3	c.1633G > A	p.Glu545Lys
		TP53	1142	34.9	c.524G > A	p.Arg175His
22	S34TP	*TP53*	551	28.3	c.641A > G	p.His214Arg
23	S35TP	*KRAS*	770	41.3	c.35G > T	p.Gly12Val
24	S36TP	*TP53*	1142	34.9	c.524G > A	p.Arg175His
25	S37TP	*PIK3CA*	59	15.3	c.1624G > A	p.Glu542Lys
26	S40TP			No mutation		
27	S42TP			No mutation		
28	S43TP	*TP53*	239	74.9	c.1024C > T	p.Arg342Ter
29	S45TP			No mutation		
30	S47TP	*CTNNB1*	4591	33.7	c.121A > G	p.Thr41Ala

3.5. Association between the genes aberrantly methylated in GCs and genes targeted by PRC2 in ES cells

The fraction of genes targeted by PRC2 in ES cells was analyzed in the genes aberrantly methylated in GCs and those unmethylated in GCs because genes methylated in GCs were reported to be associated with PRC2 target genes [33]. Using the information on the PRC2 target genes in human ES cells [44,45], it was shown that the genes aberrantly methylated in GCs consisted of a larger fraction of PRC2 target genes than those unmethylated in GCs ($P = 6.64 \times 10^{-79}$) (Supplementary Fig. 6). These results confirmed that genes aberrantly methylated in GCs were associated with genes targeted by PRC2 in ES cells.

4. Discussion

In this study, we conducted comprehensive DNA methylation analysis and extensive mutation analysis of 30 GCs, and showed (1) that the number of aberrantly methylated genes was highly variable among the 30 GCs, (2) that 19 of the 30 GCs had 24 somatic mutations of 8 different genes (*CDH1*, *CTNNB1*, *ERBB2*, *KRAS*, *MLH1*, *PIK3CA*, *SMARCB1*, and *TP53*), and (3) that the CIMP was associated with mutations of oncogenes, including *ERBB2*, *CTNNB1*, *KRAS*, and *PIK3CA*, in GCs. This is one of the first studies in which both genetic and epigenetic alterations were extensively analyzed in the same set of samples, and the association between the CIMP and mutations of oncogenes in GCs was revealed here for the first time.

A similar association has been known also in colorectal cancers, but the mechanisms for this association are still unclear. As a possible mechanism, it has been proposed (1) that cancers with the CIMP can escape senescence caused by *BRAF* mutation owing to silencing of regulators of senescence by *BRAF* mutation, such as *IGFBP7* [46,47], and (2) that overexpression of the *BRAF* mutant can induce aberrant methylation at various genes, such as *MLH1* [48]. Similar possibilities can be hypothesized in GCs. As a mechanism for methylation induction by oncogenic mutation, if this applies to GCs, there is a possibility that oncogenic mutations displace factors involved in the susceptibility to methylation induction, such as RNA polymerase II [40,49–53].

Somatic mutations of four tumor-suppressor genes, *CDH1*, *MLH1*, *SMARCB1*, and *TP53*, and four oncogenes, *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA*, were identified. Among these mutated genes, *TP53* (32%), *CDH1* (20%), *PIK3CA* (10%), *CTNNB1* (9%), *KRAS* (7%), and *ERBB2* (2%) are listed in the top 15 mutated genes in GCs in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database. In contrast, mutations of *SMARCB1* have not been identified in GCs, even by whole exome sequencing [11,12], but were identified for the first time in this study, showing the usefulness of extensive mutation analysis of known cancer-related genes. *SMARCB1* encodes a component of chromatin remodeling complex, SWI/SNF, and is mutated in malignant rhabdoid tumors [54]. In GCs, the defects of components of SWI/SNF, such as mutation of *ARID1A* [11,12] and loss of BRM expression, are known [55]. Therefore, it is considered that the dysfunction of chromatin remodeling activity plays an important role in gastric carcinogenesis.

The selection of genomic blocks heavily influenced the results of unsupervised hierarchical clustering analysis. The association between the CIMP and mutations of oncogenes was clearly observed using DNA methylation profiles of the selected 6877 and 263 blocks, and some association was observed using the methylation profiles of the 25,000 blocks with CGIs. In contrast, no association was observed using the 25,000 blocks randomly selected from all the blocks. Therefore, it is considered that the selection of biologically important probes (or genes) is required to extract meaningful information from the huge amount of data obtained by comprehensive DNA methylation analysis.

We previously found that the CIMP statuses in GCs were not associated with DNA methylation statuses in background non-cancerous mucosae, contrary to expectations [30]. The presence of the CIMP(+) GCs suggested that CGIs methylated in GCs are composed of those methylated as a result of the CIMP and those methylated in background non-cancerous mucosae.

The genes aberrantly methylated in GCs here were associated with genes targeted by PRC2 in ES cells, confirming previous reports. It has been known that genes methylated in other types of cancers are associated with genes targeted by PRC2 in ES cells [49,50,53] or normal cells [40,50–52]. A recent comprehensive analysis in GCs also revealed that genes methylated in GCs were associated with genes targeted by PRC2 in ES cells [33]. EZH2, a component of PRC2, and CBX7, a component of PRC1, are known to interact with DNA methyltransferases [56,57], and these interactions seem to be a possible mechanism of the high frequency of DNA methylation of the genes targeted by PRC2.

The prognosis of the CIMP(+) patients tended to be better than that of the CIMP(−) patients. The association between the CIMP and prognosis is highly dependent upon cancer types. For example, the CIMP is associated with poor prognosis in colorectal cancers [28], lung cancers [29], and neuroblastoma [27]. In GCs, some studies showed association with good prognosis [30,31], and others showed that with poor prognosis [32,33]. The reason why the CIMP in GCs was associated with good prognosis in some studies is unknown, but it might be possible that genes involved

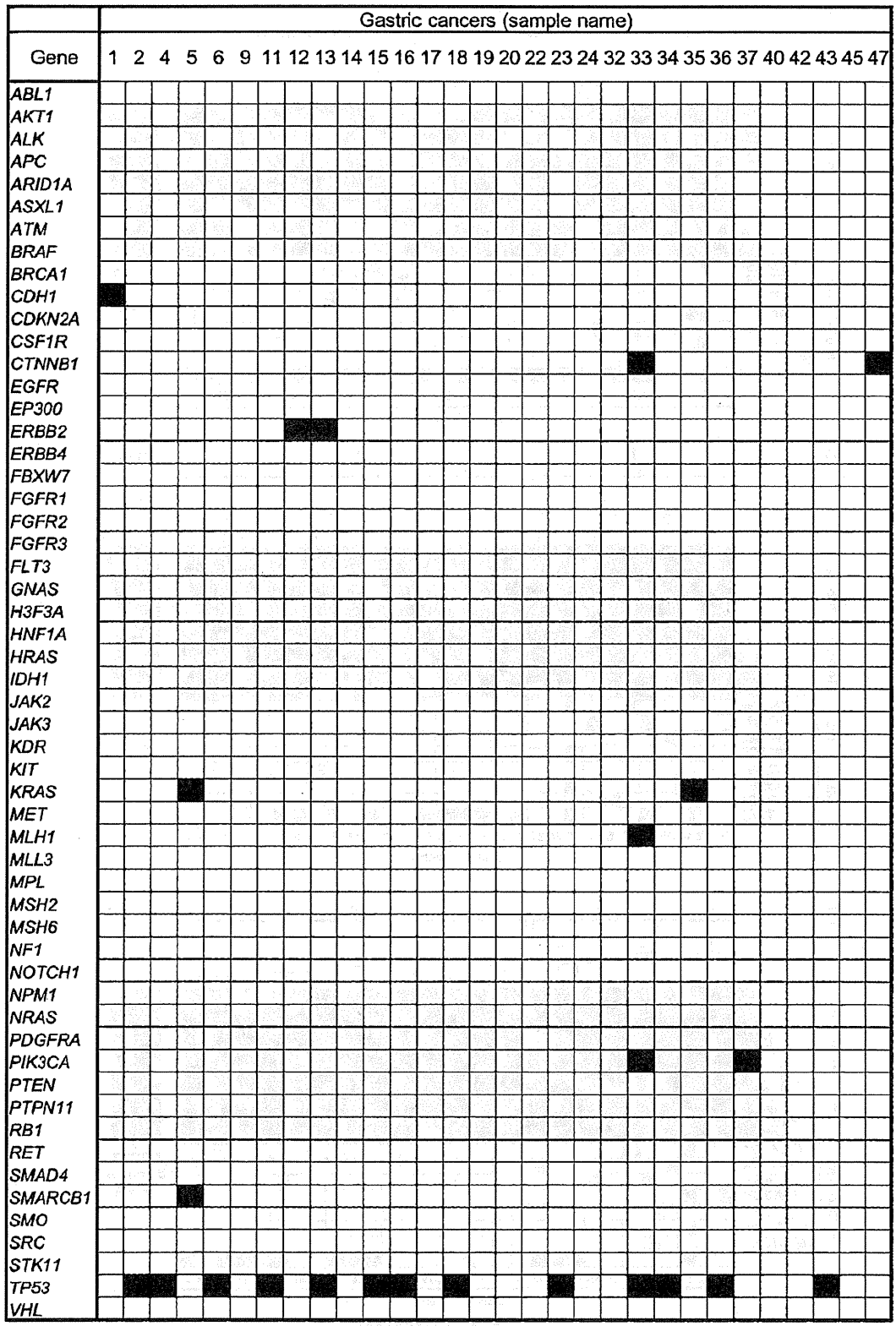

Fig. 2. Results of extensive mutation analysis of the 30 GCs. Mutations of the 55 known cancer-related genes were analyzed by Ion Torrent PGM sequencer. Among the 30 GCs, 19 had 24 somatic mutations of 8 different genes. *TP53* was mutated in 13 GCs (43%, 13 of the 30 GCs), and *CTNNB1*, *ERBB2*, *KRAS*, and *PIK3CA* were mutated in two GCs, respectively. The presence of a somatic mutation is shown by a filled square.

in tumor progression are silenced by aberrant DNA methylation in GCs with the CIMP.

In conclusion, integrated analysis of genetic and epigenetic alterations revealed that the CIMP was associated with mutations of oncogenes, including *ERBB2*, *CTNNB1*, *KRAS* and *PIK3CA*, in GCs.

Acknowledgements

This work was supported by Grants-in-Aid for the Third-Term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour and Welfare, Japan, and by the A3 Foresight Program from the Japan Society for the Promotion of Science.

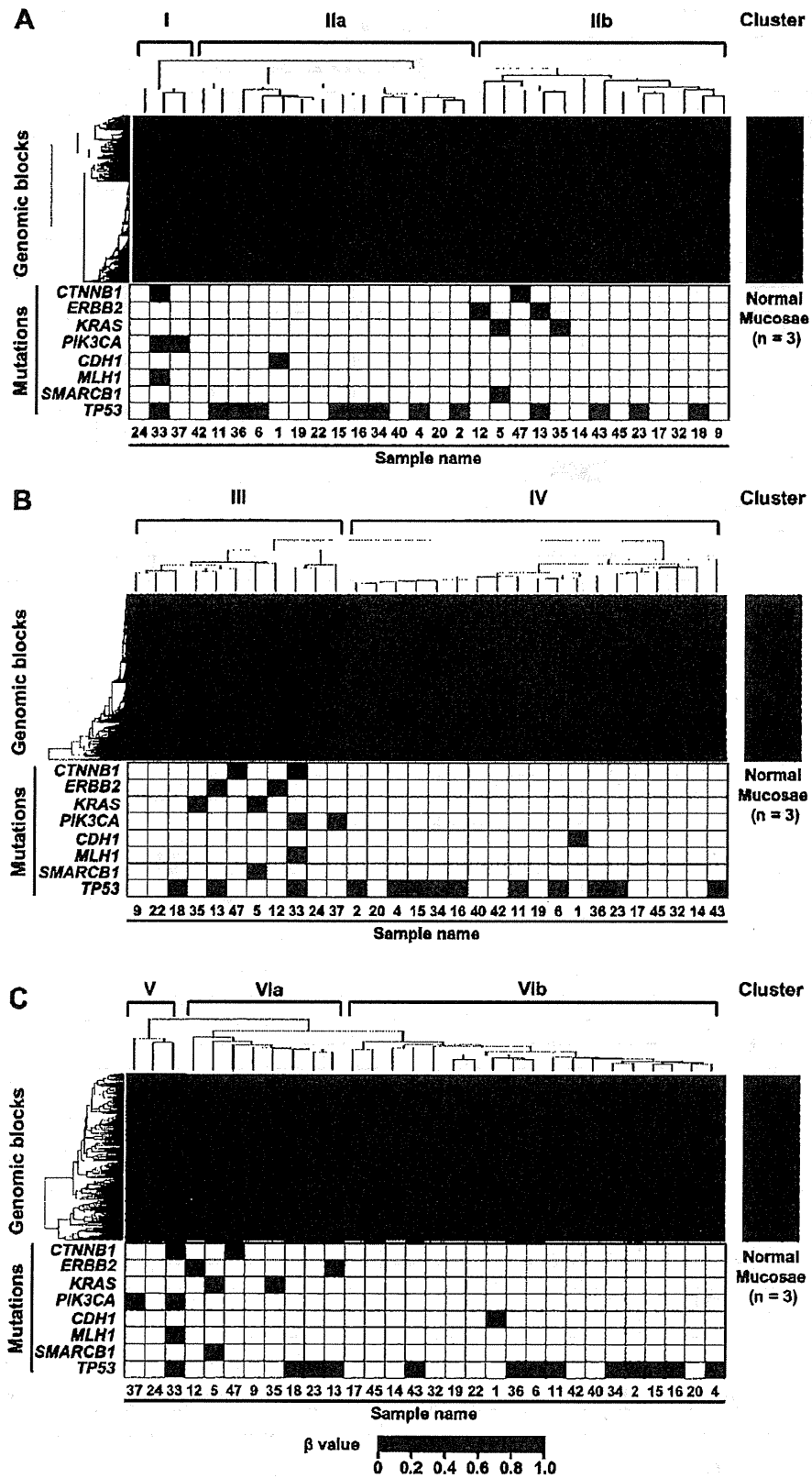

Fig. 3. The association between the DNA methylation profile and gene mutations. (A) Unsupervised hierarchical clustering analysis using DNA methylation profiles of 25,000 genomic blocks with CGIs. Clusters I ($n = 3$) and IIb ($n = 13$) contained GCs with a relatively large number of aberrantly methylated genes, and seven of the 16 GCs were shown to have mutations of oncogenes. (B) Unsupervised hierarchical clustering analysis using DNA methylation profiles of the 6877 blocks (genes) unmethylated in normal gastric mucosae. Cluster III ($n = 11$) contained GCs with a relatively large number of aberrantly methylated genes, and seven of the 11 GCs were shown to have mutations of oncogenes. (C) Unsupervised hierarchical clustering analysis using DNA methylation profiles of the 263 methylation-silenced genes. Cluster V ($n = 3$) contained GCs with the largest number of aberrantly methylated genes, and two of the three were shown to have *PIK3CA* mutations.

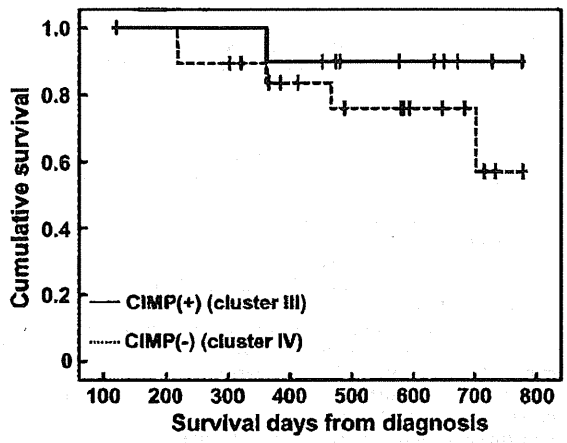

Fig. 4. The possible association between the CIMP and good prognosis. Kaplan-Meier curves were drawn using overall survival (OS). The CIMP status was determined based on the DNA methylation profile of the 6877 genes unmethylated in normal gastric mucosae. The prognosis of the CIMP(+) patients ($n = 11$) tended to be better than that of the CIMP(−) patients ($n = 19$) ($P = 0.285$).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.11.022.

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Hepatology Research

Hepatology Research 2012 doi: 10.1111/hepr.12024

Original Article

Low-dose pegylated interferon-alpha-2a monotherapy in elderly and/or cirrhotic patients infected with hepatitis C virus genotype-2 or genotype-1 low level infection

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Aim: Elderly and/or cirrhotic patients with hepatitis C virus (HCV) are at high risk of adverse effects during interferon therapy. The aim of the present study was to evaluate the efficacy, safety and predictive factors for sustained virological response (SVR) of low-dose pegylated interferon-α-2a (PEG IFN-α-2a) monotherapy in elderly and/or cirrhotic patients with HCV genotype-2 or genotype-1 low level infection.

Methods: Sixty-four elderly (≥65 years) and/or cirrhotic patients with HCV genotype-2 or genotype-1 low level (<5 logIU/mL) infection underwent low-dose PEG IFN-α-2a (90 μg/week) monotherapy for 24 weeks. Sixty patients were available for efficacy assessment.

Results: SVR was achieved in 78.3%. SVR rates according to genotype-1 low, genotype-2 low and genotype-2 high viral load were 90.0%, 87.1% and 57.9%, respectively. The discontinuation rate was 12.5%. PEG IFN-α-2a was interrupted or discontinued in four patients because of severe thrombocytopenia (<25 000/mm^3). The baseline platelet counts of all these patients were less than 70 000/mm^3. On univariate analysis of factors contributing to SVR, significant differences were noted in viral load, platelet count, γ-glutamyltransferase, ferritin, α-fetoprotein level and rapid viral response (RVR). On multivariate analysis, RVR was the only independent factor ($P = 0.010$, odds ratio = 47.27). The positive and negative SVR-predictive values based on RVR were 95% and 82%, respectively.

Conclusion: Low-dose PEG IFN-α-2a monotherapy was effective and tolerable in elderly and/or cirrhotic patients with genotype-2 or genotype-1 low HCV level infection. However, a baseline platelet count of more than 70 000/mm^3 is needed for safety. RVR can predict SVR accurately.

Key words: cirrhosis, elderly patient, genotype 2, hepatitis C virus, low viral load, pegylated interferon-α-2a

INTRODUCTION

HEPATITIS C VIRUS (HCV) elimination in cirrhotic patients leads to not only improved histological findings[1] but also to reduced progression rates to decompensated cirrhosis and hepatocellular carcinoma and improved prognosis.[2,3] By extension, HCV clearance for elderly patients after interferon (IFN) therapy can significantly reduce the risk of hepatocellular carcinoma (HCC) development and achieve prolonged survival.[4,5] Thus, as the survival benefit of HCV clearance in elderly or cirrhotic patients is reasonably larger than that in younger or non-cirrhotic patients, when the prognosis is expected to be prolonged, antiviral therapy should be performed. Because patients with HCV genotype-2 or low viral load irrespective of genotype are more sensitive to IFN than patients with genotype-1 high viral load, these patients should be treated without delay.

Current standard therapy for patients with HCV is PEG IFN and ribavirin combination therapy. However, IFN-based therapy cannot be given safely to all HCV-infected patients due to severe adverse effects. In particular, dose reduction or discontinuation of therapy due to adverse effects is more frequent in elderly or cirrhotic patients.[6–8] Many elderly patients with HCV have other comorbid diseases and cytopenia due to progressive cirrhosis. Cytopenia during IFN therapy becomes worse in such patients, and the drug often has to be reduced in dosage, interrupted or discontinued.[9] To make matters

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Received 21 September 2012; revision 9 November 2012; accepted 11 November 2012.

worse, the sustained viral response (SVR) rates tend to be lower for elderly or cirrhotic patients than for younger and non-cirrhotic patients.[10]

In Japan, standard treatment for naive patients with HCV genotype-2 high viral load is pegylated (PEG) IFN-α plus ribavirin combination therapy for 24 weeks; for patients with low viral load, conventional IFN monotherapy for 24 weeks or PEG IFN-α-2a monotherapy for 24–48 weeks is standard.[11] In practice, a reduced dose can often be given to cirrhotic patients with cytopenia to reduce the risk of severe adverse effects.[11,12] If possible, IFN treatment should be tailored on the basis of safety and prediction of efficacy in high-risk patients.

Pegylated interferon-α-2a monotherapy is more effective than IFN monotherapy[13] and better tolerated than ribavirin combination therapy, although the SVR rate is lower for PEG IFN-α-2a monotherapy than for ribavirin combination therapy.[14] However, because elderly and/or cirrhotic patients have not been included in most randomized, clinical trials, there are not enough data on the safety and efficacy of the current recommended dose of IFN-based therapy for such patients.[15] Therefore, in the present study, low-dose, shorter duration PEG IFN-α-2a monotherapy, which can decrease drug toxicity, was performed to establish an adequate and safe treatment strategy for elderly and/or cirrhotic patients who are expected to be sensitive to IFN. The aim of the present study was to evaluate the efficacy, safety and predictive factors for SVR of low-dose PEG IFN-α-2a for 24 weeks in elderly and/or cirrhotic patients with HCV genotype-2 or genotype-1 low viral load.

METHODS

Patients

THIS WAS A prospective cohort study of low-dose PEG IFN-α-2a monotherapy for high-risk patients, such as elderly and/or cirrhotic patients. A total of 64 elderly patients and/or cirrhotic patients infected with HCV genotype-2 or genotype-1 low viral load who consented to participate in this study underwent low-dose PEG IFN-α-2a monotherapy from January 2004 to December 2010 in our hospital. Patients were enrolled if any of the following were present: (i) patients were 65 years of age or older; (ii) platelet count of less than 130 000/mm^3; and (iii) presence of liver cirrhosis. The exclusion criteria were: (i) hemoglobin (Hb) levels of less than 10 g/dL; (ii) platelet count of less than 50 000/mm^3; (iii) white blood cell (WBC) count of less than 1500/mm^3 (or granulocyte count <1000/mm^3); (iv) hepatic failure or cancer; (v) patients who used *sho-saikoto* (a Kampo medicine); (vi) intractable heart disease; and (vii) uncontrollable psychoneurotic disorders. All enrolled patients underwent abdominal ultrasonography and contrast-enhanced computed tomography for diagnosis of liver cirrhosis and HCC screening within 1 month before the start of therapy. Liver cirrhosis was diagnosed clinically by imaging and laboratory tests or liver histology. A liver biopsy was performed in all patients. Even if the fibrosis grade was underestimated on liver biopsy tissue examination, liver cirrhosis was diagnosed using the morphological appearance of cirrhosis with portal hypertension, such as portosystemic shunt or hypersplenism, on imaging. Steatosis was defined as positive if hepatorenal contrast was detected on ultrasonography. In the present study, the potential benefits and risks were explained to all patients before obtaining their written, informed consent. All study protocols were approved by the ethics committee of Wakayama Medical University. The study was performed according to the World Medical Association Declaration of Helsinki.

Treatment regimens

Half of the recommended dose of PEG IFN-α-2a (Pegasys; Roche, Basel, Switzerland) was used; 90 μg PEG IFN-α-2a was administrated s.c. once a week for 24 weeks without ribavirin. There were no dose reduction criteria in this study. PEG IFN-α-2a was interrupted based on the following criteria: (i) if the Hb fell below 8.5 g/dL; (ii) if the granulocyte count fell below 500/mm^3, or the platelet count fell below 25 000/mm^3; and (iii) if deemed necessary by the attending physician because of adverse events. The treatment could be restarted if cytopenia improved. If there was no improvement in hematological parameters or adverse events within 4 weeks, this therapy was discontinued. Although there are no guidelines on the use of granulocyte colony-stimulating factor (G-CSF) for IFN-induced granulocytopenia, G-CSF (Neutrogin 100 μg; Chugai Pharmaceutical, Tokyo, Japan) was used if severe granulocytopenia (<500/mm^3) developed. If PEG IFN-α-2a was repeatedly interrupted due to granulocytopenia, G-CSF was administrated 2–3 days before weekly PEG IFN-α-2a.[16] However, erythropoietin was not allowed as supplementary treatment because the Ministry of Health in Japan had not approved its use.

Laboratory tests and liver histology

Hepatitis C virus genotype was determined using the antibody serotyping method. HCV serotype-1 and -2

correspond to genotype-1a/1b and -2a/2b, respectively. If HCV serotype could not be determined, genotype was examined. HCV RNA was measured using the quantitative and qualitative Amplicor HCV monitor ver. 2.0 test (Roche Diagnostics, Branchburg, NJ, USA) until March 2008. If HCV RNA was undetectable using quantitative reverse transcription polymerase chain reaction (RT–PCR), it was measured using qualitative RT–PCR. From April 2008, the amount of HCV RNA was measured using a COBAS TaqMan PCR assay (Roche Diagnostics). A HCV RNA level of more than 100 KIU/mL or 5 logIU/mL was defined as a high viral load, and less than 100 KIU/mL or 5 logIU/mL was defined as a low viral load. In addition to biochemical analyses including serum alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), total bilirubin, prothrombin time and albumin, levels of fibrosis markers (type IV collagen 7S and hyaluronic acid), ferritin and α-fetoprotein (AFP) were also measured within 1 month before the start of therapy. During therapy, blood cell counts were checked before treatment every week, and HCV RNA and biochemical analyses were measured every 4 weeks up to 24 weeks after the end of therapy. In all patients, within 3 months before the start of therapy, a core needle biopsy of the liver was done under ultrasound guidance using a 16-G core biopsy needle (Bard Monopty, Covington, GA, USA). The METAVIR scoring system[17] was used to analyze the histological findings and to classify patients based on activity (grades A0–A3) and fibrosis (stages F0–F4).

Assessment of effectiveness

During IFN therapy, rapid virological response (RVR) was defined as viral negativity using qualitative RT–PCR at week 4 from therapy initiation, corresponding to 1.7 logIU/mL by the TaqMan PCR assay. SVR was defined as follows: the HCV RNA was negative at the end of therapy and remained negative for 24 weeks after the end of therapy. No response was defined as detectable HCV RNA at week 24 from treatment initiation or at the end of treatment. Relapse was defined as negative at the end of therapy but positive 24 weeks after the end of therapy.

Assessment of safety and tolerability

Patients were assessed for safety and tolerability during treatment by their attending physicians who monitored adverse events and laboratory abnormalities, such as blood cell counts, every week up to week 24 and monthly thereafter. Adverse events were graded as mild (not requiring interruption or discontinuation), moderate (requiring interruption) or severe (requiring discontinuation), according to World Health Organization recommendations. The incidence and reasons for therapy discontinuation were analyzed.

Statistical analysis

Therapeutic effectiveness was determined using an intention-to-treat analysis that included patients who did not complete the scheduled course of therapy. Predictive factors for SVR were analyzed using a per protocol analysis that excluded patients who discontinued because of adverse events. The Mann–Whitney *U*-test was used to analyze continuous variables. Fisher's exact test or the χ^2-test was used to analyze categorical variables. Multivariate analysis was performed using a logistic regression model with the stepwise method. The criteria for selecting factors for multivariate analysis was $P < 0.05$. Each optimal cut-off value for continuous variables of SVR-predicting factors was decided by the Youden Index method on the basis of the receiver–operator curve (ROC). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy for SVR of RVR were calculated. Values of $P < 0.05$ were considered significant. The statistical software used was SPSS ver. 20.0J for Windows (SPSS, Tokyo, Japan).

RESULTS

Baseline background factors

THE PATIENTS' BASELINE characteristics are summarized in Table 1. There were 41 male and 23 female patients. Their median age was 68 years (range, 35–79); 45 (70.3%) patients were aged 65 years or older. Overall, 59 patients had cirrhosis; of the 45 elderly patients, 40 had cirrhosis. While 22 patients had a past history of treatment for HCC, 27 had hypertension, 15 had diabetes mellitus, 10 had diabetes mellitus and hypertension, four had thyroid dysfunction, three had chronic renal failure and three had a past history of cerebral stroke. A total of 32 patients had HCV genotype-2 low viral load, 22 had genotype-2 high viral load and 10 had genotype-1 low viral load.

Therapeutic effectiveness

Efficacy 24 weeks after the end of treatment could be assessed in 60 patients. SVR was achieved in 78.3% (47/60), relapse occurred in 16.7% (10/60) and there was no response in 5.0% (3/60). The mean PEG IFN-α-2a adherence (mean ± standard deviation) was

Table 1 Patients' baseline characteristics

Age, years (range)	68 (35–79)
<65/≥65 years (%)	19/45 (29.7/70.3)
Cirrhosis/elderly/cirrhosis and elderly	59/45/40
Sex, male/female	41/23
Bodyweight (kg)	60.4 (35.0–92.6)
Body mass index (kg/m^2)	22.4 (18.0–30.0)
Prior interferon therapy (%)	4 (6.3)
History of HCC treatment (%)	22 (34.4)
Genotype (1/2)	10/54
HCV viral load (L/H)	41/23
Genotype and viral load (1L/2L/2H)	10/32/22
White blood cells (/mm^3)	3950 (1700–7300)
Hemoglobin (g/dL)	13.0 (10.5–16.2)
Platelets (×10^4/mm^3)	9.4 (5.5–23.8)
ALT (IU/L)	45 (11–233)
GGT (IU/L)	45 (15–641)
Type IV collagen 7S (ng/mL)	8.0 (3.1–19.1)
Hyaluronic acid (ng/mL)	235.5 (29.0–1960.7)
Prothrombin time (%)	80.9 (54.2–108.0)
Albumin (g/dL)	3.7 (2.6–4.7)
Total bilirubin (mg/dL)	1.1 (0.5–2.5)
Ferritin (ng/mL)	131 (22–1034)
AFP (ng/mL)	13.7 (0.4–610.9)
Activity grade (A1/A2/A3)	22/31/11
Fibrosis stage (F1/F2/F3/F4)	0/12/30/22
Steatosis (%)	12 (18.8)
Prior splenectomy (%)	4 (6.3)

Values are expressed as medians (range) or number of patients (percent).
AFP, α-fetoprotein; ALT, alanine aminotransferase; GGT, γ-glutamyltransferase; H, high; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; L, low.

95.2% ± 18.0% in the SVR group and 95.2% ± 14.0% in the non-SVR group. There was no significant difference in PEG IFN adherence between the SVR group and the non-SVR group ($P = 0.402$). The SVR rates in the group less than 65 years old, the group aged 65–69 years old and the group aged 70 years and older were 78.9% (15/19), 78.3% (18/23) and 77.8% (14/18), respectively ($P = 0.996$). The SVR rates were 90.0% (9/10) for patients with genotype-1 low viral load, 87.1% (27/31) for patients with genotype-2 low viral load and 57.9% (11/19) for patients with genotype-2 high viral load. Relapse occurred in 10.0% (1/10) of genotype-1 low viral load, 9.7% (3/31) of genotype-2 low viral load and 31.6% (6/19) of genotype-2 high viral load. No response was seen in 0.0% (0/10) of genotype-1 low viral load, 3.2% (1/31) of genotype-2 low viral load and 10.5% (2/19) in genotype-2 high viral load. Therapeutic effectiveness according to genotype and viral load is shown in Figure 1.

Safety and tolerability

Mild adverse events occurred in seven patients; these were fatigue ($n = 4$), retinopathy with mild bleeding ($n = 2$) and psoriasis ($n = 1$). Moderate adverse events occurred in five patients. Those were thrombocytopenia ($n = 3$), acute pyelitis ($n = 1$) and granulocytopenia ($n = 1$). Therapy was discontinued in eight (12.5%) of the 64 patients. The discontinuation rates in the group less than 65 years old, the group 65–69 years old and the group 70 years and older were 10.5% (2/19), 4.3% (1/23) and 22.7% (5/22), respectively ($P = 0.168$). The reasons for therapy discontinuation due to adverse events were severe fatigue ($n = 1$), femoral neck fracture due to fall ($n = 1$), cerebral contusion due to fall ($n = 1$), thrombocytopenia ($n = 1$), severe dermatitis ($n = 2$), bacterial pneumonia ($n = 1$) and rupture of esophageal varices ($n = 1$). The ages of the two patients injured due to falls were 78 and 79 years, respectively; in both cases, the falls were accidental, and neither patient had anemia nor cerebral ischemia.

Pegylated interferon-α-2a was interrupted or discontinued in four patients because of severe thrombocytopenia (<25 000/mm^3). The baseline platelet counts of these patients were all less than 70 000/mm^3; they accounted for 40.0% (4/10) of patients with platelet counts of less than 70 000/mm^3.

Contributing factors for SVR and prediction of SVR

On univariate analysis of factors contributing to SVR, significant differences were noted in the platelet count,

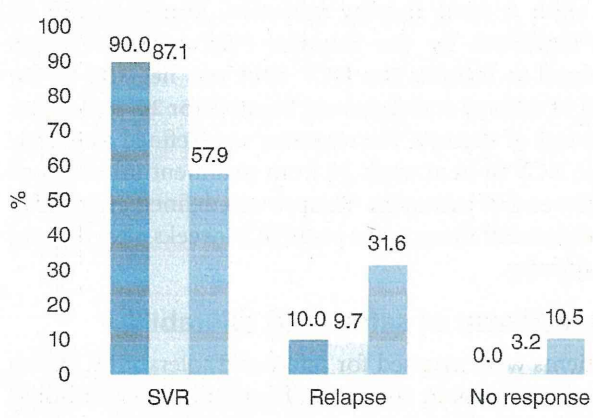

Figure 1 Therapeutic effectiveness according to genotype and viral load. SVR, sustained virological response; G1L, genotype-1 low viral load; G2L, genotype-2 low viral load; G2H, genotype-2 high viral load. ■, G1L; ■, G2L; ■, G2H.

Table 2 Comparison of factors between patients with and without SVR

Factors	SVR ($n = 44$)	Non-SVR ($n = 12$)	*P*-value
Age (years)	68	67	0.696
Sex, male/female	29/15	6/6	0.335
Bodyweight (kg)	61.0	58.0	0.516
Body mass index (kg/m^2)	22.5	22.5	0.960
White blood cells (/mm^3)	4160	3640	0.088
Hemoglobin (g/dL)	13.2	13.2	0.689
Platelets ($\times 10^4$/mm^3)	10.6	7.1	0.001
ALT (IU/L)	44	45	0.834
GGT (IU/L)	41	65	0.007
Ferritin (ng/mL)	111.9	251.9	0.021
AFP (ng/mL)	12	43.6	0.025
Type IV collagen 7S (ng/mL)	7.6	8.8	0.159
Hyaluronic acid (ng/mL)	212.6	327.0	0.168
Activity grade (A1/A2,3)	15/29	5/7	0.627
Fibrosis stage (F1,2/F3,4)	8/36	4/8	0.263
HCV RNA (logIU/mL)	4.4	5.7	0.001
Genotype (1/2)	9/35	1/11	0.671
History of HCC treatment (%)	13 (29.5)	6 (50.0)	0.185
Prior interferon therapy (%)	2 (4.5)	2 (16.7)	0.198
Steatosis (%)	9 (20.5)	3 (25.0)	0.707
RVR (positive/negative)	42/2	2/10	<0.001

Values are expressed as medians or number of patients (percent).
AFP, α-fetoprotein; ALT, alanine aminotransferase; GGT, γ-glutamyltransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; RVR, rapid virological response; SVR, sustained virological response.

GGT, ferritin, AFP level, HCV RNA load and RVR (Table 2). Cut-off values of continuous data for multivariate analysis were determined by ROC analysis as follows: HCV RNA, 5 logIU/mL; platelets, 8×10^4/mm^3; GGT, 45 IU/mL; AFP, 15 ng/mL; and ferritin, 170 ng/mL. RVR was the only independent factor on multivariate analysis (Table 3). The sensitivity, specificity, PPV, NPV and accuracy for SVR of RVR are summarized according to baseline viral load in Table 4. RVR was an accurate predictor for SVR regardless of viral load.

DISCUSSION

IN THE PRESENT study, there were some IFN-related adverse events. First, femoral neck fracture and cerebral contusion due to falls, characteristic adverse events in very elderly patients, occurred in two patients whose ages were 78 and 79 years, respectively. In our study of low-dose PEG IFN-α-2b plus ribavirin combination therapy for such high-risk patients infected with HCV genotype-1b high viral load, spinal compression frac-

Table 3 Independent factors contributing to SVR on multivariate analysis

Factors	*P*-value	Odds ratio	95% CI
HCV RNA (<5 logIU/mL)	0.289	7.02	0.19–257.73
Platelets ($>8 \times 10^4$/mm^3)	0.082	53.83	0.60–4828.20
GGT (<45 IU/mL)	0.634	2.12	0.10–46.15
Ferritin (<170 ng/mL)	0.222	11.11	0.23–527.42
AFP (<15 ng/mL)	0.324	5.65	0.18–176.70
RVR	0.010	47.27	2.49–896.31

The cut-off value for each factor was determined by receiver–operator curve analysis.
AFP, α-fetoprotein; GGT, γ-glutamyltransferase; HCV, hepatitis C virus; RVR, rapid virological response; SVR, sustained virological response.

Table 4 Sensitivity, specificity, PPV, NPV and accuracy for SVR of RVR according to viral load

Patients	Sensitivity	Specificity	PPV	NPV	Accuracy
All	95%	82%	95%	82%	92%
Low viral load	97%	75%	97%	75%	94%
High viral load	91%	86%	91%	86%	89%

NPV, negative predictive value; PPV, positive predictive value; RVR, rapid virological response; SVR, sustained virological response.

tures due to falls also occurred in two cases.[18] Therefore, elderly patients must be thoroughly warned about falls prior to IFN treatment. Other adverse events that should be monitored in elderly and/or cirrhotic patients are ruptured esophageal varices, bacterial infections and thrombocytopenia. To prevent bleeding from varices during IFN treatment, upper gastrointestinal endoscopy is performed before treatment in cirrhotic patients, and if gastroesophageal varices that have a high risk of rupture are detected, they should be treated before IFN treatment. Furthermore, Roomer *et al.*[19] reported that older patients and patients with poorly controlled diabetes mellitus have a greater risk of developing infections during HCV treatment. In the present study, acute pyelitis and bacterial pneumonia occurred in patients with diabetes mellitus and required the discontinuation of PEG IFN in the patient who had bacterial pneumonia. With respect to thrombocytopenia during treatment, the baseline platelet counts of all patients whose PEG IFN treatments were interrupted or discontinued because of severe thrombocytopenia (<25 000/mm^3) were all less than 70 000/mm^3; they accounted for 40% of the patients with platelet counts of less than 70 000/mm^3. Even this low-dose PEG IFN regimen should be avoided in patients with thrombocytopenia of less than 70 000/mm^3 for safety. Frequent monitoring for adverse events and caution with respect to unexpected fever are needed even with the low-dose regimen.

Because IFN treatment is associated with adverse effects, if SVR can be predicted even on treatment using a reduced dose and/or a shorter duration, reducing the dose or shortening the duration is desirable for safety and cost. In the present study, for high-risk patients, half of the recommended dose of PEG IFN-α-2a without ribavirin for 24 weeks was sufficiently effective (SVR rate ~90%) for patients with a low viral load irrespective of genotype and for selected patients with genotype-2 high viral load who achieved RVR. RVR is known to be the most useful predictor of SVR with standard PEG IFN plus ribavirin therapy for patients with HCV genotype 2/3 who are sensitive to IFN.[20] Using RVR, patients who can be treated for a shorter duration can be selected. With respect to PEG IFN-α-2a monotherapy, Etoh *et al.*[21] reported that PEG IFN-α-2a monotherapy using the recommended dose for 24 weeks or less may be sufficient to treat selected patients with HCV genotype 2, especially those with low viral load achieving RVR. In a small randomized trial, Iwasaki *et al.*[22] demonstrated that 24-week treatment with the recommended dose of PEG IFN-α-2a alone is clinically sufficient in patients with HCV genotype 2 and pretreatment viral load below 1000 KIU/mL who achieve RVR. Excluding patients with high titers of genotype 1 HCV, Jeong *et al.*[23] performed a prospective controlled trial to compare the efficacy of an 8-week and a 24-week course of PEG IFN-α-2a monotherapy using the recommended dose for patients negative for HCV RNA at 2 weeks after therapy initiation. Their results suggested that patients who achieved an ultra-RVR (HCV RNA negativity at week 2 from the start of therapy) can receive an 8-week course of PEG IFN-α-2a monotherapy. Thus, even for PEG IFN-α-2a monotherapy, RVR or ultra-RVR will become useful to monitor treatment efficacy and guide decisions on treatment duration. However, there are no data about whether RVR can select the patients who can be treated with a reduced dose. The present results indicated that RVR can predict SVR accurately irrespective of viral load even on low-dose PEG IFN-α-2a monotherapy for high-risk patients with genotype-2 or genotype-1 low viral load. However, the present study population was small and non-randomized. Further randomized trials in patients who achieve RVR are needed to determine the optimal dose and duration.

In conclusion, low-dose PEG IFN-α-2a monotherapy for 24 weeks was an effective and tolerable regimen for elderly and/or cirrhotic patients infected with HCV genotype-2 or genotype-1 low viral load. This low-dose regimen without ribavirin appears to be optimal in efficacy and cost for high-risk patients with low viral load irrespective of genotype. Furthermore, as an SVR of approximately 60% can be expected even for patients with genotype-2 high viral load, this regimen should

also be attempted first for naïve, high-risk patients with HCV genotype-2 high viral load. RVR is also an accurate predictor for SVR with this regimen, and it can be used to select patients who can be treated with a reduced dose. This low-dose regimen may be sufficient to treat selected patients with genotype-2 high viral load achieving RVR. However, even with this regimen, a baseline platelet count of more than 70 000/mm^3 is needed for safety, and elderly/cirrhotic patients should be warned about and carefully monitored for severe adverse events such as severe cytopenia, infections and falls.

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Digestive Endoscopy 2012; ••: ••–••

doi: 10.1111/den.12017

Original Article

Usefulness of a continuous suction mouthpiece during percutaneous endoscopic gastrostomy: A single-center, prospective, randomized study

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Background: No mouthpiece has been designed to control salivary flow during endoscopic procedures. A new continuous suction mouthpiece (CSM) was developed, and its usefulness for percutaneous endoscopic gastrostomy (PEG) was evaluated.

Patients and Methods: Seventy-two patients who were scheduled to undergo PEG or the exchange of a gastrostomy button or tube were assigned to one of two groups: the group using the CSM and the group using the conventional mouthpiece. Aspiration pneumonia, procedure duration, extent of salivary flow, frequency of saliva suction, and number of choking episodes during the procedures were evaluated and compared between the two groups.

Results: The same number of patients was randomly allocated to each group. There were no significant differences between the two groups in sex, age, procedure type, duration of procedure, depth of sedation, and indication for the procedure. The grade of salivary flow was significantly lower in patients with the CSM than in patients with the conventional mouthpiece ($P < 0.001$). Significantly fewer suctions and choking episodes were observed in patients with the CSM than in patients with the conventional mouthpiece ($P = 0.013$, and $P = 0.015$, respectively). Aspiration pneumonia and other significant adverse events were not observed in either group.

Conclusions: CSM reduced the number of episodes associated with salivary flow in PEG-related procedures. The device is expected to reduce complications such as aspiration not only in PEG but in other upper endoscopic procedures.

Key words: aspiration, mouthpiece, percutaneous endoscopic gastrostomy, salivary flow

INTRODUCTION

PERCUTANEOUS ENDOSCOPIC GASTROSTOMY (PEG) is a procedure that was developed to provide direct access to the stomach with endoscopy in 1980,[1] and it has been increasingly carried out for patients who suffer from dysphagia and/or need long-term enteral nutrition. As PEG is relatively safe and easy to carry out, it has become the preferred option for creating a gastrostomy.

However, there is a non-negligible risk of adverse events in association with PEG. In particular, aspiration is the most problematic, because PEG is carried out with the patient in the supine position, it takes a long time, and it is likely to be carried out in elderly patients with dysphagia. Moreover, sedation during the procedure may increase the risk. The reported rate of early phase complications in PEG is approximately 2.3%.[2]

One of the most important factors correlated with aspiration is salivary flow induced by the introduction/extraction of the endoscope into the oral cavity. In this context, control of salivary flow during PEG is important for the prevention of aspiration. However, few attempts have been made to control salivary flow, perhaps due to its difficulty. Currently, an endoscopist or an assistant must check the accumulation of saliva and suction it using a catheter in case the patient undergoing the procedure cannot discharge saliva from the mouth.

During the upper endoscopy procedure, a hard plastic mouthpiece is used to protect the endoscope from being bitten and for smooth insertion of the endoscope without

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Received 3 September 2012; accepted 6 November 2012.

hindrance from the tongue. A mouthpiece having the function of suctioning saliva might be useful for preventing aspiration during endoscopic procedures. However, no mouthpiece has been designed for this purpose.

With this background, a new continuous suction mouthpiece (CSM) was developed to prevent adverse events in association with salivary flow during endoscopy. The mouthpiece was made by hand by retrofitting a commercially available mouthpiece. The aim of the present study was to evaluate the usefulness and the ability of the newly developed CSM for the prevention of aspiration during PEG by examining salivary flow and the incidence of aspiration-related events during the procedure.

METHODS

Equipment

AFTER CUTTING THE junction part of a non-toxic polyvinyl chloride (PVC) suction tube (NIPRO SUCTION CATHETER® 14-Fr; NIPRO, Osaka, Japan), the tube was bent double, and each part was connected with two movable short bands made of non-toxic PVC suction tube (NIPRO SUCTION CATHETER® 16-Fr; NIPRO). The three parts divided by the short bands were made into: a 2–5-cm-diameter loop intra-oral part for suction; a binding loop part to fit mouthpieces of various sizes; and an extra-oral part having two ends, both of which were linked to the Y-shaped connector (ARAM, Osaka, Japan) (Fig. 1A). The Y-shaped connector could be connected to the main suction tube leading to the suction unit (Shin-Ei Industries, Tokyo, Japan).

Smooth, 2.7-mm-diameter holes were made spirally in six locations in the body of the intra-oral loop, at equally spaced intervals. The holes were arranged in such a way as to prevent direct suction of the oral mucosa. The size of the intra-oral loop can be changed from 2 to 5 cm by sliding movable short bands to match the physique of each patient. Finally, the MB-142 mouthpiece (Olympus, Tokyo, Japan) was inserted into the binding loop part. The finished product of the CSM is shown in Figure 1B,C.

For the upper endoscopic procedure, the patient was placed on his or her left side and asked to bite down on the mouthpiece with the intra-oral loop with holes placed inside the left cheek (Fig. 2). Suctioning was continuously carried out at low pressure (10 kPa) through the unification tube attached to the Y-shaped connector during the endoscopic procedure. For the control subjects, the MB-142 mouthpiece was used as it was.

Patients and study design

This was a single-center, prospective, randomized, controlled study. Patients with brain disease, neurological

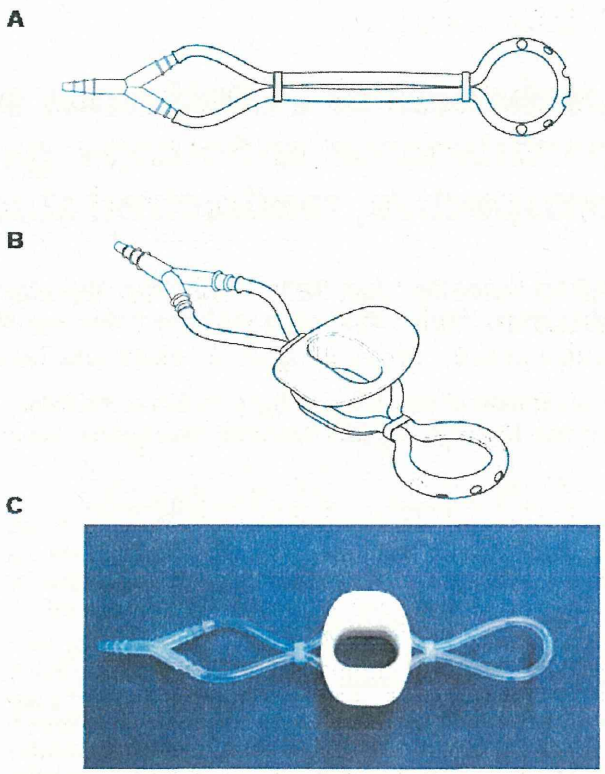

Figure 1 Continuous suction mouthpiece (CSM). (A) CSM without the mouthpiece. (B) CSM with the mouthpiece. (C) Photograph of the CSM.

disease, dementia, temporomandibular joint disorder, disuse syndrome, or a psychiatric disorder who were scheduled to undergo PEG or the exchange of a gastrostomy button or tube with endoscopy at Nakaya Hospital (Wakayama, Japan) from March 2011 to December 2011 were recruited. Patients were excluded if they had a history of respiratory problems that could increase the risk of complications associated with aspiration pneumonia and salivary flow. Eligible patients were randomly assigned to each of the following groups: the group using the CSM and the group using the conventional mouthpiece for the procedure. Randomization was carried out using the sealed envelope technique. During the procedures, salivary flow and complications associated with aspiration were evaluated and compared between the two groups. By its nature, this study could not be blinded.

This study was approved by the ethics committee of Nakaya Hospital. Written, informed consent was obtained from each patient or the next of kin. We have registered this study into University hospital Medical Information Network (UMIN) as UMIN000008575. The CSM was developed by our institute without any financial or equipment support from companies.