RESEARCH COMMUNICATION

Methylation of Secreted Frizzled Related Protein Gene in Acute Leukemia Patients in China

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Abstract

Background: DNA methylation of CpG islands within the promoters of specific genes may play roles in tumor initiation and progression. It has been suggested such events may serve as critical check points. Methods:The present study analyzed the methylation status of CpG islands within the promoters of secreted frizzled-related proteins (SFRPs) in 87 acute leukemia (AL) patients, 20 normal controls, and four AL cell lines. 5-aza-2'-deoxycytidine (5-Aza-CdR), an inhibitor of DNA methylation, was employed to determine its effect on SFRP expression. Result: Methylation of at least one SFRP promoter was observed in 69% of the AL patients analyzed. In addition, methylation of all four SFRP promoters was observed in Molt-4, Jurkat, HL60 and NB4 cells. In Jurkat cells, methylation levels of four SFRP promoters decreased in a dose-dependent manner upon treatment with 5-Aza-CdR, which coincided with increased mRNA expression. With increasing 5-Aza-CdR concentrations, the expression of DNA methyltransferases, DNMT3A and DNMT3B, significantly decreased in a dose-dependent manner. Conclusion: The present study demonstrated that SFRP gene methylation may be involved in AL progression, with a possible epigenetic mechanism influencing Wnt signaling.

Keywords: Acute leukemia - CpG islands - DNA methylation - secreted frizzled-related proteins

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Introduction

Several studies have examined the presence of DNA methylation of CpG islands in leukemia. For example, Chung et al. (2002) reported that methylation of the tumor suppressor gene, Adenomatous polyposis coli (APC), was present in more than 50% of patients with T cell leukemia or lymphoma. In acute lymphoblastic leukemia (ALL), promoter hypermethylation is associated with poor prognosis (Roman-Gomez et al., 2007). Furthermore, in the T cell leukemia cell line, ST1 cells, APC gene expression was recovered after treatment with 5-aza-2'-deoxycytidine (5-Aza-CdR), a methylation blocker (Yang et al., 2005). Thus, Chung et al. hypothesized that methylation of the APC gene might be associated with β-catenin overexpression in acute T lymphoblastic leukemia (Chung et al., 2002). In addition, cell lines with methylation-induced Dickkopfs-3 (DKK-3) gene silencing were extremely sensitive to the Wnt signaling (Roman-Gomez et al., 2004).

Aberrant activation of Wnt/β-catenin signaling is thought to play an important role in tumorigenesis and progression in breast cancer (Chung et al., 2004). Aberrant Wnt signaling may be a result of a mutation in the β-catenin gene, inactivation of tumor suppressor genes, as well as methylation-associated silencing of Wnt pathway inhibitor genes, including secreted frizzled-related proteins (SFRPs) (Kawano and Kypta, 2003).

SFRPs, extracellular antagonist of Wnt signaling, inhibit Wnt signaling in both classical and nonclassical means (Kawano and Kypta, 2003). In addition, SFRPs block aberrant Wnt activation, promoting tumor cell apoptosis. Although genetic changes in the SFRP gene have not been observed in tumors, the promoters of SFRP1-2, -4 and -5 consistently contain CpG islands (Esteller et al., 2001). In a study of 20 patients with chronic B lymphocyte leukemia, methylation-related silencing of the SFRP gene was common; the methylation ratios of SFRP-1, -2, -4 and -5 were 100%, 55%, 30% and 15%, respectively (Liu et al., 2006). However, the silencing of SFRP-1, -2 and -5 is also frequently observed; therefore, methylation may be not the only contributing factor.

The present study aimed to investigate the role of aberrant methylation of CpG islands in the promoters SFRP genes in patients with acute leukemia (AL) as well as four AL cell lines. Real-time RT-PCR was employed to detect SFRP-1, -2, -4 and -5 mRNA expression in Jurkat cells with and without 5-Aza-CdR treatment. Because DNA methyltransferases (DNMTs) may be involved in possible SFRP methylation, the effects of 5-Aza-CdR on DNMT3A and DNMT3B gene expression were assessed. If DNA methylation of SFRP genes is involved in AL, it could be a biomarker for AL initiation and progression as well as treatment efficacy.

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Materials and Methods

Human tissue samples

From 2006 to 2009, a total of 87 patients with a confirmed diagnosis of AL were enrolled consecutively from the Department of Hematology of the Union Hospital Affiliated with the Fujian Medical University. The diagnosis of AL was based on the French-American-British (FAB) guidelines (Bennett et al., 1985). Another 20 healthy volunteers or patients without hematologic malignancies served as controls. The bone marrow cells or peripheral blood were collected from all AL patients and healthy controls. Informed consent was obtained for each patient, and the study was approved by the Ethics Committee of the Fujian Medical University.

Cell culture and 5-aza-2'-deoxycytidine (5-Aza-CdR) treatment of the Jurkat cell line

AL cell lines, including Molt-4 cells and Jurkat cells, the acute myelogenous leukemia (AML) cell line, HL60 cells, and the acute promyelocytic leukaemia (APL) cell line, NB4 cells, were purchased from the Fujian Institute of Hematology (Fuzhou city, Fujian, China). All cell lines were maintained in RPMI1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco). To determine the effects of 5-Aza-CdR (Sigma, St. Louis, MO), Jurkat cells were incubated with 1.0, 2.0, and 4.0 μmol/L for 72 h.

Sodium bisulphite treatment and methylation-specific polymerase chain reaction (MSP)

The bone marrow cells were used for analysis; however, if they were not available peripheral blood was used for MSP analysis. Genomic DNA was extracted from the patient samples and cell lines using the Wizard DNA Clean-Up System (Promega, WI, USA); 2 μL of DNA were used as template for MSP. DNA bisulfite modification was detected according to the methods described by Herman and Baylin (2003). The primers for specific methylated and unmethylated genes were designed according to Suzuki et al. and shown in Table 1 (Suzuki et al., 2004). RKO cell DNA served as a positive control as the methylation of SFRP has been previously reported (Suzuki et al., 2004), and ddH2O served as a blank control. DNA extract from the peripheral blood of healthy subjects served as negative controls. After MSP, the products were then analyzed using 2% agarose gel electrophoresis. To validate the reliability of detecting SFRP methylation by MSP, products following amplification were randomly selected and subjected to T-A cloning and sequencing. The sequence was then compared with wild-type.

Real-time reverse transcription polymerase chain reaction

Total DNA was extracted from Jurkat cells using TRIzol (Invitrogen, CA, USA) and then reverse transcribed into first strand cDNA using M-MLV Reverse Transcriptase (Promega). Semi-quantitative PCR was performed using the Platinum SYBR Green I qPCR SuperMix-UDG kit (Invitrogen) in a 7500 thermocycler (Applied Biosystems, Foster City, CA). Table 1 presents the primer sequences used GAPDH served as an internal reference, and relative mRNA expression of SFRP-1 and -2, after demethylation was determined using the ^{2}-ΔΔCT method.

Reverse transcription polymerase chain reaction

RT-PCR was carried out in 7500 thermocycler (Applied Biosystems) using Platinum SYBR Green I qPCR SuperMix-UDG kit (Invitrogen). GAPDH served as an internal reference, and the optical density of target genes was normalized to that of GAPDH to determine the relative expression of target genes.

Statistical analysis

Subjects' characteristics were summarized as n (%) in sex and compared between AL patients and healthy controls using Pearson Chi-square test between group. Age was shown as median with range and compared using the Mann-Whitney U test. Furthermore, frequency of methylation in patients was presented as a bar charts. Expression of DNMT3A and DNMT3B genes in Jurkat cells were shown as mean±standard deviation (SD) and compared among concentrations using one-way ANOVA with post-hoc Bonferroni adjustments. P-values <0.05 were considered significant. P-values <0.01 (0.05/4) were considered significant for the Bonferroni adjustments among the following concentrations 0, 1.0, 2.0, and 4.0 μmol/L. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS Inc, Chicago, IL, USA).

Results

Patient characteristics and SFRP gene methylation status

The characteristics of the 87 AL patients and 20 healthy control participants were shown in Table 2. No significant

Table 1. Sequences for Primers Used in this Study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
Real-time RT-PCR			
SFRP1	TCTCTGTGCCAGCGAGTTTG	TCAGGTCCTTCTTCTTGATGGG	128
SFRP2	ACGACATAATGGAAACGCTTTG	TGGTCTTGCTCTTGGTCTCC	114
SFRP4	TGTTGACTGTAAACGCCTAAGC	GGGATGGGTGATGAGGACTTG	192
SFRP5	CCCCTGGACAACGACCTCTG	GACCACAAAGTCACTGGAGCAC	147
GAPHD	CCCCTTCATTGACCTCAACTACAT	CGCTCCTGGAAGATGGTGA	135
RT-PCR			
DNMT3A	CACACAGAAGCATATCCAGGAGTG	AGTGGACTGGGAAACCAAATACC	551
DNMT3B	AATGTGAATCCAGCCAGGAAAGGC	ACTGGATTACACTCCAGGAACCGT	190
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG	493

RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

100.0
6.3
75.0
56.3
50.0

Table 2. Characteristics of the Patient Cohort

Variables	AL patients (n=87)	Healthy controls (n=20)	P-value
Sex, males (%)	52 (59.8)	8 (40.0)	0.108
Age, years	35 (14 – 80)	30 (14 – 71)	0.764
FAB subtypes			
AML			
M0	1 (0.9)	–	
M1	5 (4.7)	–	
M2	17 (15.9)	–	
M3	7 (6.5)	–	
M4	0 (0)	–	
M5	26 (24.3)	–	
M6	3 (2.8)	–	
M7	0 (0)	–	
ALL			
L1	10 (9.3)	–	
L2	11 (10.3)	–	
L3	7 (6.5)	–	

Data were shows as n(%) for sex and FAB subtypes and median with range for age; AL, Acute leukemia; FAB, French-American-British; AML, Acute myelogenous leukemia; ALL, acute lymphoblastic leukemia

differences in age and gender were observed between the AL patients and the healthy control group. Separating the AL patients by FAB subtype revealed 26 (24.3%) in M5, 17 (15.9%) in M2, 5 (4.7%) in M1, 3 (2.8%) in M6, and 1 (0.9%) in M0 for AML whereas 11 (10.3%) in L2, 10 (9.3%) in L1 and 7 (6.5%) in L3 for ALL (Table 2). Because no SFRP promoter methylation as observed in healthy controls, this suggests that this step may be involved in AL initiation or progression.

As shown in Figure 1 and Table 3, promoter hypermethylation of the SFRP1, -2, -4, and -5 genes in samples obtained from AL patients was assessed. Representative images of the MSP analysis were shown in Figure 1A. The frequency of promoter methylation for each SFRP gene was shown in Figure 1B; 31% AL patients had no methylated SFRP gene, 36% had one, 17% had two, and 3% had three genes. The distribution of patients according to the SRFP gene that was methylated was also determined (Figure 1C); 35.6% (31/87), 25.3% (22/87), 12.6% (11/87), and 17.2% (15/87) of 87 AL patients had methylated SFRP1, SFRP2, SFRP4, and SFRP5 promoters, respectively.

Methylation status of the SFRP gene promoter regions in acute leukemia cell lines

The methylation status of SFRP gene promoters was assessed in Molt-4 cells and Jurkat AL cell lines, the AML cell line, HL60 cells, and the APL cell line, NB4 cells (Figure 2). Presents the methylation analysis of SFRP1, -2, -4, and -5 in four AL cell lines. Methylation

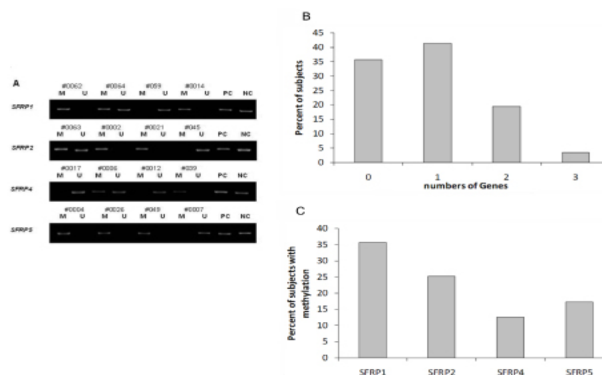

Figure 1. Promoter Hypermethylation of SFRP1, -2, -4, and -5 in Samples From Patients with Al. (A) Representative MSP analysis of methylated (M) and unmethylated (U) SFRP1, -2, -4, and -5 in patients with AL at diagnosis. DNA from the peripheral blood of healthy controls served as the negative control (NC), while DNA from the RKO cell line served as positive controls (PC). (B) Distribution of patients according to the number of methylated genes. (C) Frequency of methylated SFRP gene promoters in AL patients.

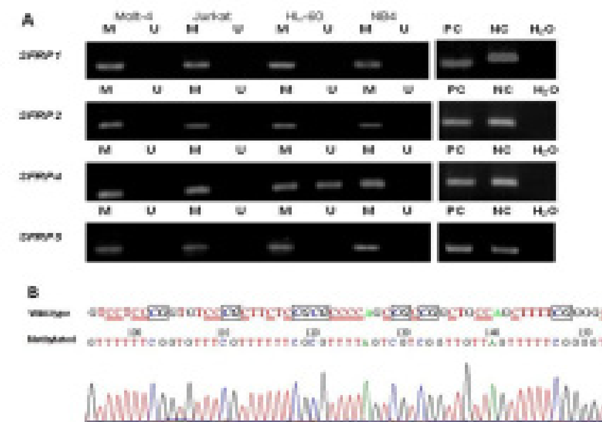

Figure 2. Methylation Analysis of SFRP1, -2, -4, and -5 in Four AL Cell Lines. (A) MSP analysis of methylated (M) and unmethylated (U) SFRP1, -2, -4, and -5 sequences in Molt-4, Jurkat, HL-60, and NB4 cell lines. DNA from the peripheral blood of healthy controls served as the negative control (NC), while DNA from the RKO cell line served as positive controls (PC). (B) SFRP2 DNA sequencing of methylated MSP product, showing conversion of C to T after bisulphite treatment, but not in the CpG island

of the promoters of all analyzed SFRP promoter region was detected in the positive control, RKO cell line, but not in the no DNA (ddH_2O) controls (Figure 2A). Methylation of all four SFRP promoters was observed in all four cells lines (Figure 2A). As shown in Figure 2B, SFRP2 DNA sequencing of M-MSP product was undertaken; conversion of C to T after bisulphite treatment was observed. However, the CpG island remained

Table 3. MSP Analysis of SFRP1,-2,-4 and -5 in the AL Patients

	SFRP1 methylation	SFRP2 methylation	SFRP4 methylation	SFRP5 methylation
AL patients (n=87)	31 (35.6%)	22 (25.3%)	11 (12.6%)	15 (17.2%)
ALL (n=28)	11 (39.3%)	7 (25.0%)	7 (25.0%)	8 (28.6%)
AML (n=59)	20 (33.9%)	15 (25.4%)	4 (6.8%)	7 (11.9%)
Healthy controls	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Data were summarized as n(%); AL, Acute leukemia; AML, Acute myelogenous leukemia; ALL, acute lymphoblastic leukemia

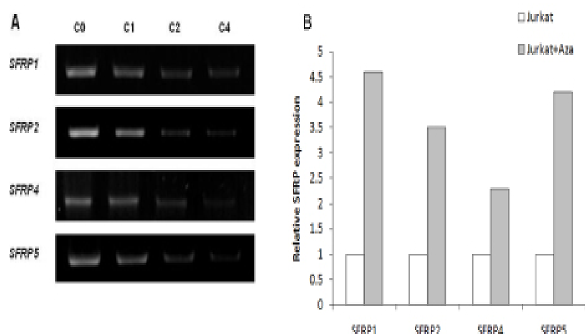

Figure 3. Methylation and Expression Analysis of SFRP Genes in AL Cell Line Jurkat. (A) MSP analysis in Jurkat cells of the four SFRP genes without and with 1.0 μmol/L (C1), 2.0 μmol/L (C2), and 4.0 μmol/L (C4) 5-Aza-CdR (Aza) for 72 h. (B) Relative changes in SFRP1, -2, -4, and -5 expression levels with and without treatment with 4.0 μmol/L Aza for 72 h. Expression levels of SFRP1, -2, -4, and -5 were determined by real-time RT-PCR in Jurkat cells treated with and without Aza. GAPDH served as a housekeeping gene to which SFRP genes were normalized

unchanged. Furthermore, with exception of the SFRP4 promoter region in HL-60 cells that was heterozygous for methylation, all other analyzed SFRP promoters in all four cell lines were methylated at both alleles .

To determine if methylation of SFRP promoters coincided with decreased expression, expression analysis of the SFRP genes was undertaken in the presence and absence of the demethylation agent, 5-Aza-CdR in Jurkat cells (Figure 3). Specifically, Jurkat cells were treated for 72 h with 1.0 μmol/L (C1), 2.0 μmol/L (C2), and 4.0 μmol/L (C4) 5-Aza-CdR (Figure 3A). With increasing 5-Aza-CdR concentrations, the methylation levels of SFRP-1, -2, -4 and -5 decreased. Relative changes in SFRP1, -2, -4, and -5 expression levels before and after 4.0 μmol/L 5-Aza-CdR treatment for 72 h (Figure 3B). 5-Aza-CdR treatment increased SFRP1, 2, 4, and 5 mRNA expression by 4.6-, 3.5-, 2.3-, and 4.2-fold, respectively. These results suggest that promoter methylation downregulates SFRP gene expression in Jurkat cells.

Expression of the DNMT3A and DNMT3B genes in Jurkat cell line

Expression analysis of DNMT3A and DNMT3B genes in response to 5-Aza-CdR treatment in Jurkat cells was assessed (Figure 4). With increasing 5-Aza-CdR concentrations, the expression of DNMT3A and DNMT3B significantly decreased in a dose-dependent manner (Figure 4A and 4B; P<0.01). in the average expression of DNMT3A relative to GAPDH was 0.72±0.01, 0.66±0.02, 0.28±0.02,and 0.05±0.01 for 0.0, 1.0, 2.0, and 4.0 μmol/L 5-Aza-CdR, respectively. For DNMT3B, the average relative expression level was observed 0.61±0.02, 0.58±0.02, 0.26±0.01,and 0.06±0.01 for 0.0, 1.0, 2.0, and 4.0 μmol/L 5-Aza-CdR, respectively.

Discussion

DNA methylation is a critical check point in tumorigenesis (Herman and Baylin, 2003); therefore, it also represents a possible therapeutic target for many

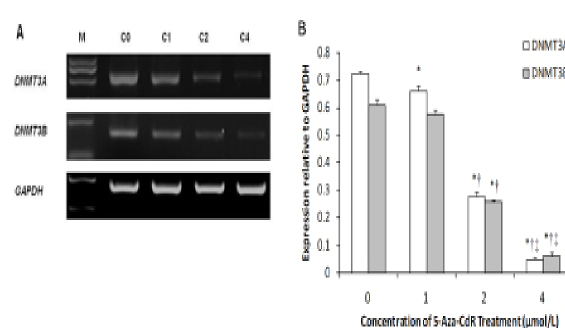

Figure 4. Effects of 5-Aza-CdR (Aza) Treatment on DNMT3A and DNMT3B Gene Expression in Jurkat Cells. (A) Semi-quantitative RT-PCR analysis of the DNMT3A and DNMT3B genes in Jurkat cells with and without 1.0 μmol/L (C1), 2.0 μmol/L (C2), and 4.0 μmol/L (C4) Aza for 72 h. M, DNA marker. (B) Expression levels of DNMT3A and DNMT3B determined using semi-quantitative RT-PCR in Jurkat cells following treatment with aza for 72 h at various concentrations (1.0, 2.0 and 4.0 μmol/L); GAPDH expression was used as an internal control. Data were shown as mean±standard deviation (SD) and compared among concentrations using one-way ANOVA with post-hoc Bonferroni adjustments. P<0.01, significantly different as compared with *0 μmol/L, †1.0μmol/L, and ‡ 2.0μmol/L

tumors, including leukemia. Because SFRPs are involved in lung and colorectal cancers, the methylation status of SFRP promoters was evaluated in AL patients and healthy controls in the present study. Whereas SFRP promoters were unmethylated in healthy controls, methylation of at least one SFRP promoter was observed in 69% of the AL patients analyzed.

Methylation and subsequent inactivation of SFRP genes has been reported for multiple cancers, including hepatocellular carcinoma (Takagi et al., 2008), gastric cancer (Nojima et al., 2007) multiple myeloma (Jost et al., 2009), and leukemia (Liu et al., 2006). Jost et al. (2009) detected methylation of the SFRP gene in 76 multiple myeloma (MM) patients at their first treatment. Specifically, hypermethylation of SFRP-1, 2, 4 and 5 was observed in 35.5%, 52.6%, 1.3% and 6.9% of patients, respectively. Furthermore, hypermethylation of SFRP-5 was closely related to the development of disease (Jost et al., 2009). In 20 patients with chronic B-lymphocytic leukemia, methylation-induced silencing of the SFRP gene was commonly observed, and the methylation ratio of SFRP-1, -2, -4 and -5 was 100%, 55%, 30% and 15%, respectively (Liu et al., 2006). In the present study, methylation of SFRP-1, -2, -4 and -5 was observed in AL patients; however, its relationship with disease status was not undertaken, but will be the subject of future studies.

SFRP genes serve as a “gateway” gene for the Wnt signaling pathway. Under normal conditions, SFRP expression effectively suppresses aberrant activation of Wnt signaling; however, suppression by SFRP is lost upon methylation (Cadigan and Liu, 2006). In myeloma cell lines and primary myeloma tissues, aberrant activation of Wnt signaling in LP1 and WL2 cells was associated with methylation-related silencing of several Wnt inhibitor genes (Chim et al., 2007). Expression of these Wnt inhibitor genes were recovered by demethylation, subsequently reducing Wnt signaling. In primary MM

patients, 42% of patients had methylation of at least one inhibitor gene, and 61.9% had the methylation of more than two genes (Chim et al., 2007). In the present study, expression of SFRP genes was increased upon 5-Aza-CdR treatment, suggesting that promoter methylation inhibits their expression. The effects of this inhibition on Wnt signaling will be analyzed in future studies.

In view of the relationship between DNA methylation and certain cancers, demethylation therapy has been a focus for possible treatments. Treatment with demethylation agents has been used in hematologic malignancies, including myelodysplastic syndrome (MDS) (Qiu et al., 2010). 5-Aza-CdR, an epigenetic cytotoxic drug approved by the U.S. Food and Drug Administration (FDA), blocks entrance into S phase. Phosphorylated 5-Aza-CdR, a nucleoside analogue, forms covalent interactions with DNA methyltransferase (DNMT), inhibiting their interaction with DNAT, resulting in demethylation (Jones and Taylor, 1980). Thus, it was speculated that 5-Aza-CdR may inhibit DNMT expression as well as inhibit the transfer of methyl groups to CpG islands. In the present study, treatment with 5-Aza-CdR for 72 h increased SFRP expression, which coincided with decreased methylation in the Jurkat cells. Furthermore, DNMT3A and DNMT3B mRNA expression was dose-dependently downregulated by 5-Aza-CdR. Although these data suggest that 5-Aza-CdR may be useful for AL, it should be noted that its demethylation effect is not cell-specific. Thus, 5-Aza-CdR not only corrects aberrant methylation associated with tumorigenesis, but also activates the expression of other genes normally suppressed, resulting in disordered gene regulation. Therefore, the clinical application of 5-Aza-CdR must be further evaluated in clinical trials.

The present study has limitations that warrant further discussion. For example, the relationship between SFRP methylation and AL disease status was not assessed. Therefore, this will be the subject of future studies. In addition, the effects of SFRP methylation on Wnt signaling were not evaluated.

Taken together, the present study demonstrated methylation of SFRP gene promotes in AL patients. Further studies are required to investigate the epigenetic mechanism of SFRP gene methylation in the AL as well as its influence on Wnt signaling as well as patient outcomes. SFRP gene methylation may represent a potential biomarker for the early diagnosis of AL and/or prognosis and therapeutic efficacy of various treatments.

Acknowledgements

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