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Therapeutic response to Paroxetine in major depressive disorders predicted by DNA methylation

Naohiro Takeuchi [a], Shinpei Nonen [a], Masaki Kato [b], Masataka Wakeno [b], Yoshiteru Takekita [b], Toshihiko Kinoshita [b], Fumihiko Kugawa [a]

[a] School of Pharmacy, Hyogo University of Health Science, Hyogo, Japan

[b] Department of Neuropsychiatry, Kansai Medical University, Osaka, Japan

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Short title

Paroxetine response and DNA methylation

All correspondence should be addressed to Dr. Shinpei Nonen.

Department of Pharmacy, Hyogo University of Health Sciences, Hyogo, Japan

1-3-6 Minatojima, Chuo-ku, Kobe, 650-8530, Japan

Tel: +81-78-304-3149, Fax: +81-78-304-2849

E-mail: nonen@huhs.ac.jp

Abstract

Background: Antidepressants have variable therapeutic effects, depending on genetic and environmental factors. Approximately 30% of major depressive disorder (MDD) patients do not respond significantly to antidepressants such as Paroxetine, a selective serotonin reuptake inhibitor (SSRI). However, the biological mechanisms behind this phenomenon are mostly unknown. Here we examined the role of patients' epigenetic background in SSRI effectiveness. **Methods:** Genome-wide DNA methylation analysis of the peripheral blood of Japanese MDD patients was performed by using the Infinium HumanMethylation450 BeadChip. **Results:** We compared the results of the 10 patients who best responded to Paroxetine (BR) with the 10 worst responders (WR), and found 623 CpG sites with a >10% difference in DNA methylation level. Among them, 218 sites were nominally significant between BR and WR ($p < 0.05$), and 2 sites (cg00594917 and cg07260927) were significantly different after False Discovery Rate (FDR) correction ($q < 0.05$). The methylation difference was greatest at cg00594917, located in the first exon of the PPFIA4 gene, which codes for Liprin-α ($p=0.00012$). Hierarchical cluster analysis of 23 CpG sites in the PPFIA4 gene distinguished BR and WR except for 1 WR patient. The cg07260927 site was located in the 5'UTR of the Heparin sulfate-glucosamine 3-sulfotransferase 1 (HS3ST1) gene ($p=0.00013$). Hierarchical cluster analysis of 28 CpG sites in HS3ST1 distinguished BR and WR except for 1 WR and 2 BR patients. **Conclusion:** Our results suggest that patients' DNA methylation profile at specific genes such as PPFIA4 and HS3ST1 is associated with individual variations in therapeutic responses to

Paroxetine.

Introduction

Major depressive disorder (MDD) is a serious health problem and global burden on society [1, 2]. The lifetime prevalence of MDD in Japan is estimated to be 6.5% [3]. Selective serotonin re-uptake inhibitors (SSRIs) such as Paroxetine do not affect 30% of MDD patients [4]. This low efficacy is explained in part by inter-individual genetic differences [5]. Candidate genes responsible for the pharmacological effectiveness of SSRIs include serotonergic pathway components, because SSRIs block the serotonin transporter (5-HTT) at its first step of action. 5-HTT has several functional polymorphisms, including the 5-HTT linked promoter region (5-HTT LPR) and 5-HTT variable number tandem repeat, located in the upstream regulatory region and the second intron, respectively [6, 7]. We previously reported that 5-HTT LPR and rs25531, a functional polymorphism near 5-HTT LPR, are associated with the variability of the clinical response to SSRIs [8, 9].

DNA methylation is considered to be an essential epigenetic mechanism for regulating gene expression [10]. Methylation of the brain-derived neurotrophic factor (BDNF) gene is a candidate epigenetic biomarker for diagnosing depression disorder [11]. Likewise, hypermethylation of the serotonin transporter gene (SLC6A4) is a candidate biomarker for bipolar disorder based on a study using discordant monozygotic twins [12]. Besides these studies, in which DNA methylation was taken advantage for prognosis of psychiatric disorders, Domschke and Powell were also interested

in DNA methylation for prediction of clinical response of antidepressants [13, 14].

Although reports have suggested that fluctuations in DNA methylation regulate the pathogenesis of psychiatric disorders, little is known about the biological mechanism by which antidepressants combat MDD, especially the role of DNA methylation. Here, using the blood of Japanese MDD patients who received Paroxetine for their routine treatment, we performed a comprehensive DNA methylation analysis and compared patients who responded well to the drug to those who did not.

Materials and methods

Subjects and treatment

Sixty-eight MDD patients who were receiving Paroxetine treatment through Kansai Medical University Hospital were subjects in this study. These patients had participated in another pharmacogenomic study conducted by Kato et al. [15], and the same blood specimens were used in the present study. This study was approved by the ethical committees of Hyogo University of Health Sciences and Kansai Medical University, and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained after a detailed explanation of the study. The diagnosis of major depression was confirmed by the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). Any subject 1) with a clinically significant unstable medical illness, 2) with pregnancy, 3) in whom a principle psychiatric disorder other than major depression was diagnosed, 4) with a history of substance abuse or dependence active within the previous 6 months except for

nicotine dependence, or 5) treated with electroconvulsive therapy within the previous 6 months, was excluded. MDD was diagnosed by two independent senior psychiatrists and confirmed by a third psychiatrist, blind to the previous evaluations.

The psychological and physiological conditions of the patients were evaluated by the 21-item Hamilton Rating Scale for Depression (HAM-D21) [16]. The HAM-D21 assessment was performed before (baseline) and 2, 4, and 6 weeks after Paroxetine administration. The trained senior psychiatrists who evaluated the scores were all blind to the genetic data of this study. The patients were confirmed to be drug-free. Patients taking antidepressants were ordered to undergo a ten-day washout period. Paroxetine was administered at an initial dose of 20 mg/day, and increased to a therapeutic dose of up to 40 mg/day. The increasing dosage of Paroxetine started on day 8 or 11 and continued until the end of trial. During the experimental term, concomitant psychotropic drugs were not allowed, except for sleep-inducing hypnotic agents (equivalent doses to Diazepam 3.67 ± 4.11 mg/day) at bedtime. The percent improvement in HAM-D scores was calculated using the equation: [(baseline score – score at week 6) × 100 / baseline score]. Among the 68 patients, the 10 who showed the greatest improvement in HAM-D score were defined as best responders (BR), and the 10 who showed the least improvement were defined as worst responders (WR).

Genomic DNA extraction and methylation analysis

Genomic DNA was extracted from the patients' peripheral leukocytes using the QIAamp Blood

Kit according to the manufacturer's instructions (Qiagen, Tokyo, Japan). The DNA methylation levels were evaluated using the Illumina Infinium HumanMethylation450 BeadChip (HM450) (Illumina, Tokyo, Japan). HM450 interrogates 485,764 CpG sites covering 99% of the Reference Sequence database genes, with an average of 17 CpG sites per each gene. In general, the methylated sites are mapped across the promoter region, 5'UTR, first exon, open reading frame, and 3'UTR of each gene. HM450 also includes 96% of the CpG Islands (CGI) that are registered in the University of California Santa Cruz (UCSC) database, with additional coverage of the CGI shores (0-2 kb from the CGI) and CGI shelves (2-4 kb from the CGI). Detailed information about HM450 is available in the HM450 User Guide, from the Illumina Corporation. (www.illumina.com).

The DNA methylation data were analyzed using IBM SPSS Statistics 22.0 software. The DNA methylation level of individual CpG sites was calculated using the equation: [signal from a methylated probe / (signal from a methylated probe + signal from its unmethylated counterpart)]. This calculated value, known as β, ranges from 0 (completely unmethylated) to 1 (fully methylated). Nakamura, Yuen, and Córdova-Palomera proposed that a difference in methylation between 2 groups is significant if it is more than 10% [17-19]. We adopted this assumption in the present study, setting a threshold at 10%; that is, a difference in β between BR and WR (Δβ) above 10% ($|\Delta\beta| \geq 0.1$) was defined as a significant difference. The CpG sites on the X and Y chromosomes, polymorphisms within 10 bp of the target CpG site, and sites of poor quality data (signal intensity of the output raw data from genome-wide methylation analysis using HM450 < 500) were excluded.

Statistical analysis

The ages, baseline HAM-D21 scores, percent improvement in HAM-D21 scores, methylation levels of each site, and Paroxetine dosage after six weeks of medication between the BR and WR groups were evaluated by independent t-tests. A chi-square test was used to analyze the significant differences in sex. Significance was set at $p < 0.05$. For the multiple testing correction via the False Discovery Rate (FDR) [20], the threshold was set at $q < 0.05$. Hierarchical cluster analyses were performed using IBM SPSS Statistics 22.0 software.

Results

Patient demographics

The patient characteristics are shown in Table 1. No significant differences were found for age, sex, HAM-D21 baseline score, or Paroxetine dosage after six weeks of medication between the BR and WR groups. A significant difference was only found for the percent improvement in HAM-D21 score after 6 weeks Paroxetine treatment ($p < 0.001$). All BR achieved remission, while no WR remitted.

Comparative analysis of DNA methylation profiles

DNA methylation analysis using HM450 revealed 623 differentially methylated sites ($|\Delta\beta| \geq 0.1$).

Among them, 218 sites were nominally significant between the BR and WR groups ($p < 0.05$). We placed the P values of the 218 CpG sites in ascending order, and focused on the 10 sites with the lowest P values (Top10). The P values for Top10 ranged from 0.000124 to 0.00152. Seven of the Top10 sites were located in intragenic regions (including the gene body or its regulatory region) and three were in intergenic regions (Table 2).

Hierarchical cluster analysis of the Top10 sites

We conducted a hierarchical cluster analysis of the Top10 sites (Fig. 1). The results were expressed as a heat map indicating the methylation level of each CpG site, and as a dendrogram (Fig. 1, left). The dendrogram in Fig. 1 clearly indicated that the BR and WR groups were distinguished from each other by the Top10 methylation levels, except for 2 BR patients.

Hierarchical cluster analysis of the PPFIA4 and HS3ST1 genes

Among the Top10 sites, two (cg00594917 and cg07260927) showed statistical significance even after FDR correction ($q < 0.05$), so we analyzed them further. The difference in methylation level was the most significant at cg00594917, located in the first exon of the PPFIA4 gene, which codes for Liprin-α ($p=0.00012$). The second site, cg07260927, was located in the 5'UTR of the Heparin sulfate-glucosamine 3-sulfotransferase 1 (HS3ST1) gene ($p=0.00013$).

We next examined the association between the clinical response to Paroxetine and the methylation

levels of the above 2 genes (PPFIA4 and HS3ST1) by hierarchical cluster analyses (Fig. 2 and 3). Fig. 2 shows that the methylation state of 23 CpG sites in the PPFIA4 gene distinguished the BR and WR groups except for one WR patient. Similarly, the methylation of 28 CpG sites in the HS3ST1 gene distinguished the two groups except for one WR and two BR patients (Fig. 3).

Next, we performed independent t-tests to analyze the difference in the methylation ratio at individual CpG sites of PPFIA4. The methylation rates of 6 of the 23 CpG sites in PPFIA4 were statistically significant ($p < 0.05$) between the BR and WR groups (6 stars above the heat map in Fig. 2). At all 6 of these sites (one site was in the first exon and 5 were in the gene body), the methylation levels of the WR group were higher than those of the BR group. From upstream to downstream, the $\Delta\beta$ values of the 6 starred sites were 10.5%, 3.5%, 1.3%, 5.8%, 3.8% and 15.3%, respectively.

We conducted a similar analysis for the HS3ST1 gene. The methylation rates of 5 of the 28 CpG sites in HS3ST1 were statistically significant ($p < 0.05$) between the two groups (5 stars above the heat map in Fig. 3). In 4 of the 5 sites (one in TSS200 in the downstream region and 3 in the 5'UTR), the methylation levels of the WR group were higher than those of the BR group. From upstream to downstream, the $\Delta\beta$ values of the 5 starred sites were -1.8%, 0.9%, 4.3%, 4.6% and 14.3%, respectively.

Discussion and Conclusion

In the present study, we conducted comprehensive DNA methylation analyses to investigate the

correlation between clinical response to Paroxetine and DNA methylation levels. A total of 623 CpG sites showed more than a 10% difference in DNA methylation between the BR and WR groups. Among these 623 sites, 218 were nominally significant between the two groups ($p < 0.05$), and the Top10 sites were selected based on the 10 lowest P values. Hierarchical cluster analyses based on the individual patients' methylation levels distinguished the two groups from each other. After FDR correction, two CpG sites (cg00594917 and cg07260927) showed statistical significance; they were also the top 1 and 2 sites among the Top10 (Table 2).

Liprin-α, the product of the PPFIA4 gene, is related to neural signal transmission. Previous reports showed that 1) Liprin-α is critical for proper neural transmission at the presynaptic nerve terminal [21], 2) Liprin-α can interact with the glutamate receptor interacting protein (GRIP) [22], and 3) muscarinic acetylcholine receptor-dependent long-term depression involves interactions between Liprin-α and GRIP [23]. This information is compatible with the possibility that the PPFIA4 gene, which contains the cg00594917 site, affects Paroxetine's pharmacological effect on MDD patients. Since 23 CpG sites were mapped to the PPFIA4 gene, we focused on these sites for further analysis.

By hierarchical cluster analysis of these sites, the BR and WR groups were almost completely distinguished (Fig. 2). Interestingly, higher methylation levels were indicated in the WR group than the BR group at all 6 significant sites. Five of the 6 sites were located in the gene body of PPFIA4. In general, the DNA methylation of a promoter region is considered to be critical for gene expression; however, some reports suggest that methylation of the gene body also plays a role in controlling gene

expression [24, 25]. Therefore, a difference in the methylation level of the PPFIA4 gene body may affect a patient's therapeutic response to Paroxetine.

The other site, cg07260927, was located in the 5'UTR of the HS3ST1 gene. This gene encodes a member of the heparin sulfate biosynthetic enzyme family; it possesses heparin sulfate conversion activity and is a rate-limiting enzyme for heparin biosynthesis [26, 27]. We also conducted the hierarchic cluster analysis for 28 CpG sites in HS3ST1 mapped in HM450. As shown in Fig. 3, this analysis distinguished the BR and WR groups except for 1 WR and 2 BR patients. To our knowledge, there is no concrete evidence that HS3ST1 can affect the pharmacological effects of Paroxetine or its interaction with neural network elements. However, our results suggest some hidden potential of this gene; specifically, the methylation of the CpG sites in HS3ST1 might affect the onset or progression of MDD as well as the therapeutic effect of Paroxetine on MDD patients.

Among the other genes listed in Table 2, several studies suggest that, like PPFIA4, ATP binding cassette subfamily A member 13 (ABCA13) and MicroRNA 659 (miR-659) are related to central nervous system (CNS) diseases. ABCA13, a member of the ABC superfamily, has been identified as a cause of cytogenetic abnormality. Furthermore, rare coding variants of ABCA13 are responsible for schizophrenia, bipolar disorder, and depression [28].

MiR-659 is reported to bind the mRNA of progranulin (GRN), which is related to frontotemporal dementia (FTLD-U). A common genetic variant (rs5848), located in the 3'UTR of GRN in a binding-site for miR-659, significantly increases the risk of developing FTLD-U via a suppressed translation

of GRN [29]. These findings support the possibility that the methylation of ABCA13 and miR659 could affect the clinical response to Paroxetine.

The biological functions of the other genes listed in Table 2, LEMD2, PAX8, and ELL2, have also been reported. The LEM domain containing 2 (LEMD2) gene encodes a nuclear membrane protein that is critical for nuclear envelope formation [30]. The Paired box 8 (PAX8) gene, a member of the PAX family, is a transcription factor that is important for multicellular organism development [31, 32]. RNA polymerase II, 2 (ELL2) is a transcription elongation factor that directs immunoglobulin secretion in plasma cells by stimulating altered RNA processing [33].

The Top10 methylation sites in Table 2 can be divided into three groups. First, three genes (PPFIA4, ABCA13, and miR-659) form a group for which it is easy to ascertain their possible connection to the neural physiology of MDD patients. The second group consists of genes (HS3ST1, LEMD2, PAX8, and ELL2) for which we have little information about their physiological connection to the pharmacological effects of Paroxetine in MDD patients. The third group includes three methylation sites on inter-genetic regions or sites not registered as an identified gene. In this study, our hierarchical cluster analysis indicated that some methylation sites on PPFIA4 and HS3ST1 could be important for explaining the inter-individual differences of Paroxetine efficacy. However, we note that the results obtained here were almost all based on computational methodologies. Thus, further “wet” experiments are needed to confirm that the methylation of these genes is key to the pharmacological effect of Paroxetine.

There are several limitations in the present study. 1) The sample size was 10 each for the BR and WR groups. Further studies with a larger sample size are needed to confirm these findings. 2) HM450 was the ideal tool for the purpose of this study; however, the chip does not cover every CpG site in the genomic DNA. 3) We used DNA samples prepared from patients' peripheral blood; notably, however, several studies have reported that the DNA methylation patterns are highly correlated between the blood and brain [34]. 4) The BR and WR were defined as the percent improvement in HAM-D scores only, while other clinical symptoms not considered in this study may be relevant, such as the number of failed trials of other antidepressants and the presence or absence of individual residual symptoms after Paroxetine treatment. Even though we lack experimental evidence, we think it is likely that the DNA methylation of the peripheral blood of our patients closely reflected its counterpart DNA in the brain.

In conclusion, we revealed that DNA methylation of the PPFIA4 and HS3ST1 genes is associated with the therapeutic responses of MDD patients to Paroxetine. To our knowledge, this is the first study to reveal a connection between DNA methylation and the pharmacological effects of Paroxetine.

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Disclosure Statement

The authors declare no conflict of interest.

Figure legends

Fig. 1. Hierarchical cluster analysis for the Top10 methylation sites

Hierarchical cluster analysis for the Top10 methylation sites shown in Table 2 was conducted. At the top of the heat map, 10 methylation sites are indicated by their RefGene-Name (UCSC). IG1, IG2, and IG3 stand for InterGenic regions 1, 2, and 3, respectively. IG1 refers to Probe ID cg01056004; IG2 to Probe ID cg17611235, and IG3 to Probe ID cg12765123. The methylation levels of each gene are depicted by a pseudo color scale. The dendrogram at left almost divided the 20 patients into two groups. The upper part (above the horizontal line) corresponds to the BR group and the lower part (below the horizontal line) corresponds to the WR group, with two exceptions belonging to the BR group.

Fig. 2. Hierarchical cluster analysis of the PPFIA4 gene

Hierarchical cluster analysis of the 23 CpG sites in the PPFIA4 gene was conducted. The meanings of TSS1500, TSS200, 1st exon, and 3'UTR are shown in the legend for Table 2. Stars (☆) indicate CpG sites whose P values showed statistical significance by an independent t-test (see text for details). The dagger (†) indicates the site of cg00594917, shown in Table 2. The methylation levels of each site are depicted by a pseudo color scale. The dendrogram at left almost divided the 20 patients into two groups. The upper part (above the horizontal line) corresponds to the BR group with one exception belonging to the WR group, and the lower part (below the horizontal line)

corresponds to the WR group.

Fig. 3. Hierarchical cluster analysis of the HS3ST1 gene

Hierarchical cluster analysis of the 28 CpG sites in the HS3ST1 gene was conducted. The meanings of TSS1500, TSS200, 1st exon, 5'UTR, and gene body are shown in the legend for Table 2. Stars (☆) indicate CpG sites whose P values showed statistical significance by an independent t-test (see text for details). The dagger (†) indicates the site of cg07260927, shown in Table 2. The methylation levels of each site are depicted by a pseudo color scale. The dendrogram at left almost divided the 20 patients into two groups. The upper part (above the horizontal line) corresponds to the BR group with one exception belonging to the WR group, and the lower part (below the horizontal line) corresponds to the WR group with two exceptions belonging to the BR group.

Table 1 Patient characteristics

Characteristic	BR (n = 10)	WR (n = 10)	P value
Age (years: Mean ± SD)	52.7 ± 18.5	47.0 ± 11.0	0.413
Sex (Male/Female)	5/5	2/8	0.160
HAM-D baseline score (Mean ± SD)	21.0 ± 6.00	22.9 ± 5.51	0.47
Improvement in HAM-D score (%: Mean ± SD)	95.2 ± 4.77	27.7 ± 22.3	< 0.001
Dosage after six weeks of medication (mg: Mean ± SD)	24.4 ± 5.27	30.0 ± 6.67	0.062

HAM-D: Hamilton depression rating scale, BR: Best responders, WR: Worst responders

Table 2 Top10 ranking of differentially methylated CpG sites between BR and WR

Probe ID	RefGene-Name (UCSC)	RefGene-Group (UCSC)	Chromosome	β (BR)	β (WR)	Δβ	P value
cg00594917	PPFIA4	1stExon	1q32.1	0.761	0.866	0.105	0.00012$
cg07260927	HS3ST1	5'UTR	4p16	0.353	0.495	0.143	0.00013$
cg13859433	LEMD2	3'UTR	6p21.31	0.356	0.457	0.101	0.00055
cg01056004	*	*	10	0.280	0.474	0.194	0.00055
cg17611235	*	*	11	0.639	0.747	0.108	0.00102
cg09704166	PAX8	Body	2q13	0.538	0.365	-0.173	0.00107
cg12765123	*	*	10	0.402	0.634	0.232	0.00118
cg15541008	ELL2	1st Exon	5q15	0.369	0.264	-0.105	0.00138
cg20307184	ABCA13	Body	7p12.3	0.743	0.627	-0.116	0.00139
cg07059402	miR659	TSS1500	22q13.1	0.406	0.571	0.165	0.00152

Probe ID: Illumina probe ID for individual target site,
Two sites, cg00594917 and cg07260927, showed statistical significance ($) after FDR correction.
BR: Best responders, WR: Worst responders, β: methylation level, Δβ: β (WR) - β (BR).
Asterisks indicate that the methylation sites were located in an intergenic region, 5'UTR: 5' untranslated region,
3'UTR: 3' untranslated region, TSS: transcription start site, Body: gene body, TSS1500: within 1500 bp of TSS
PPFIA4: Protein tyrosine phosphatase, receptor type, f polypeptide, interacting protein (liprin), alpha 4
HS3ST1: Heparin sulfate-glucosamine 3-sulfotransferase 1, LEMD2: LEM domain containing 2, PAX8: Paired box 8
ELL2: Elongation factor, RNA polymerase II, 2, ABCA13: ATP binding cassette subfamily A member 13,
MIR659: MicroRNA 659

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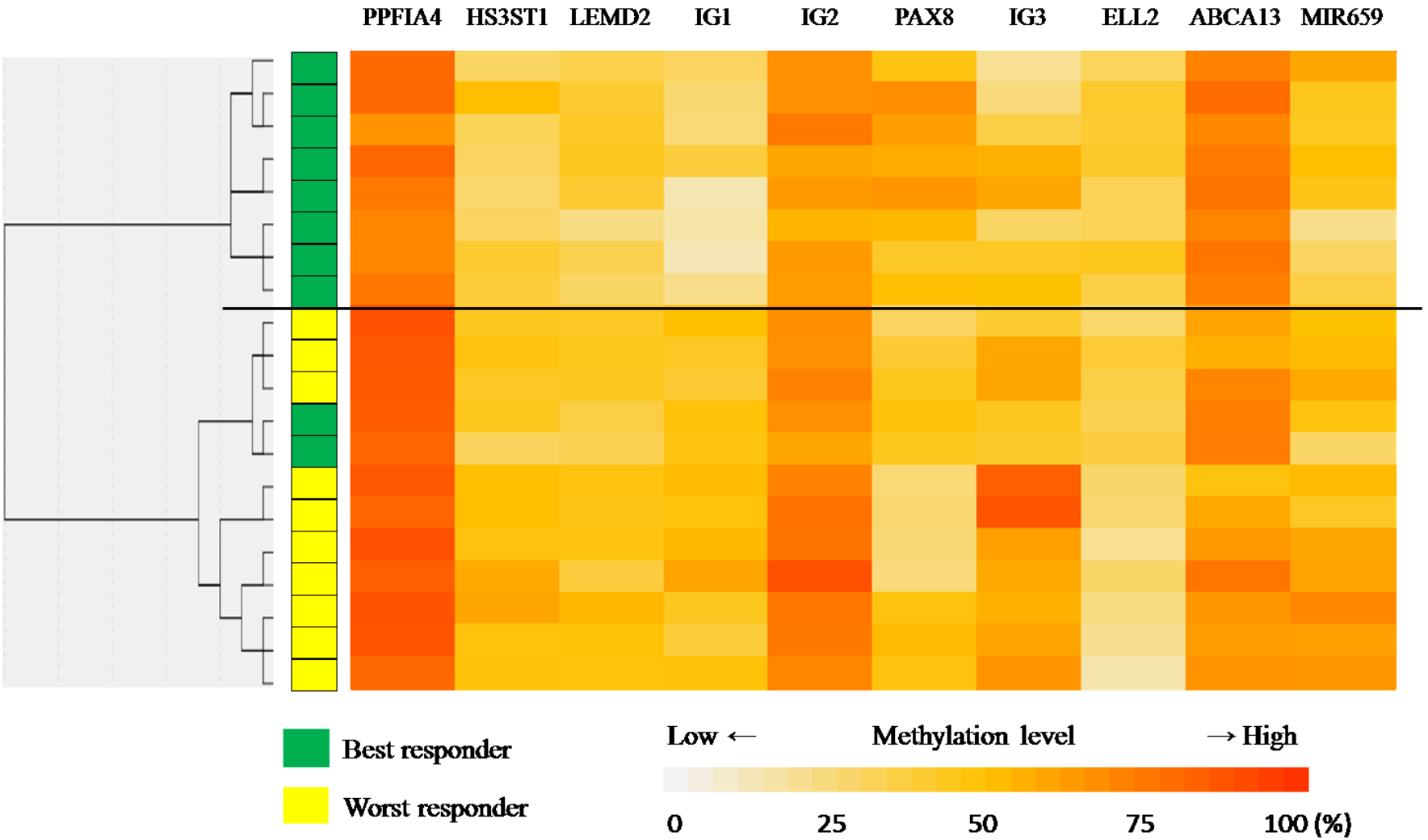
PPFIA4
HS3ST1
LEMD2
IG1
IG2
PAX8
IG3
ELL2
ABCA13
MIR659
Best responder
Worst responder
Low ←
Methylation level
→ High
0
25
50
75
100 (%)

Fig. 1.

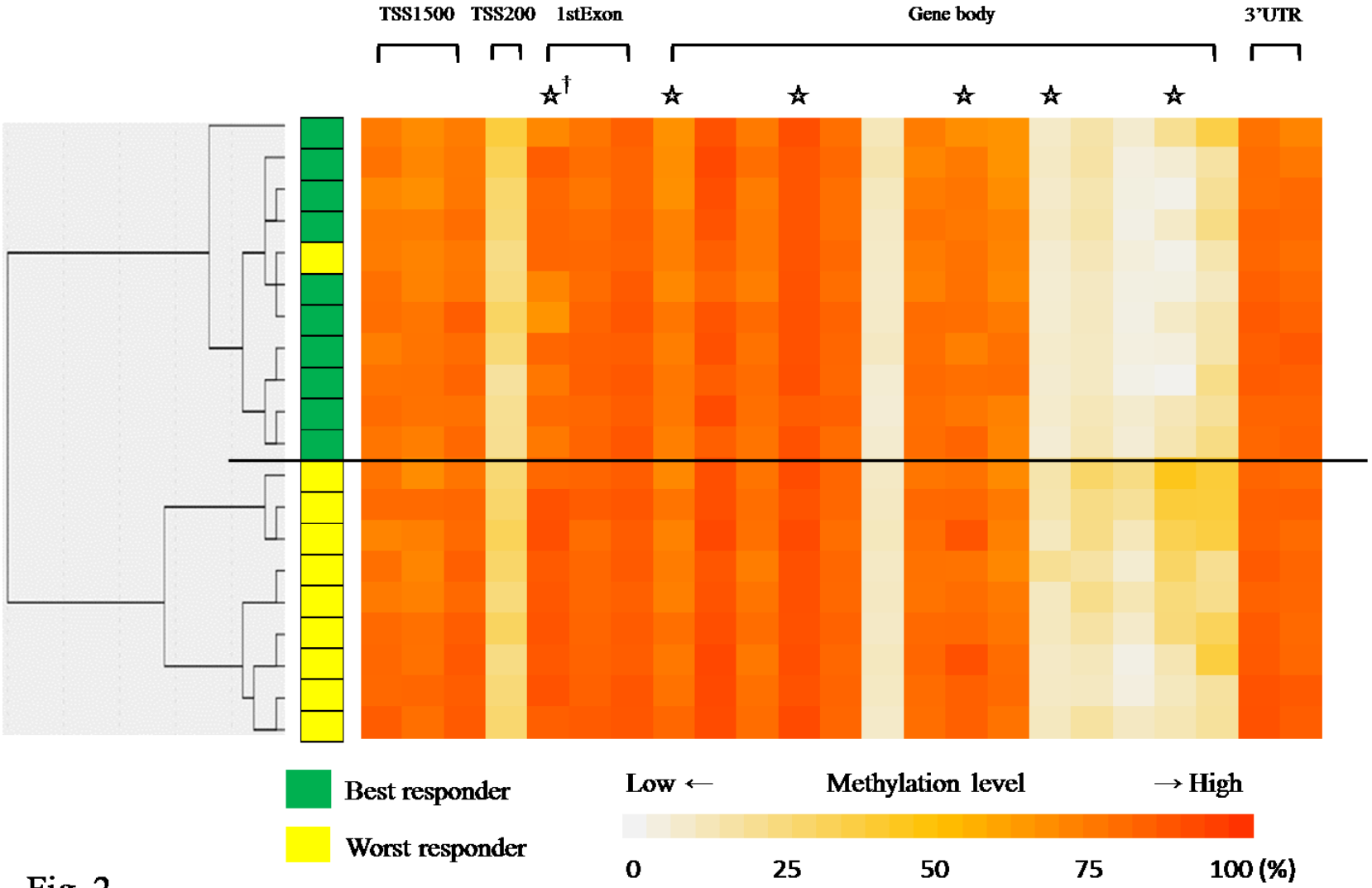
TSS1500
TSS200
1stExon
Gene body
3'UTR
Best responder
Worst responder
Low ← Methylation level → High
0
25
50
75
100 (%)

Fig. 2.

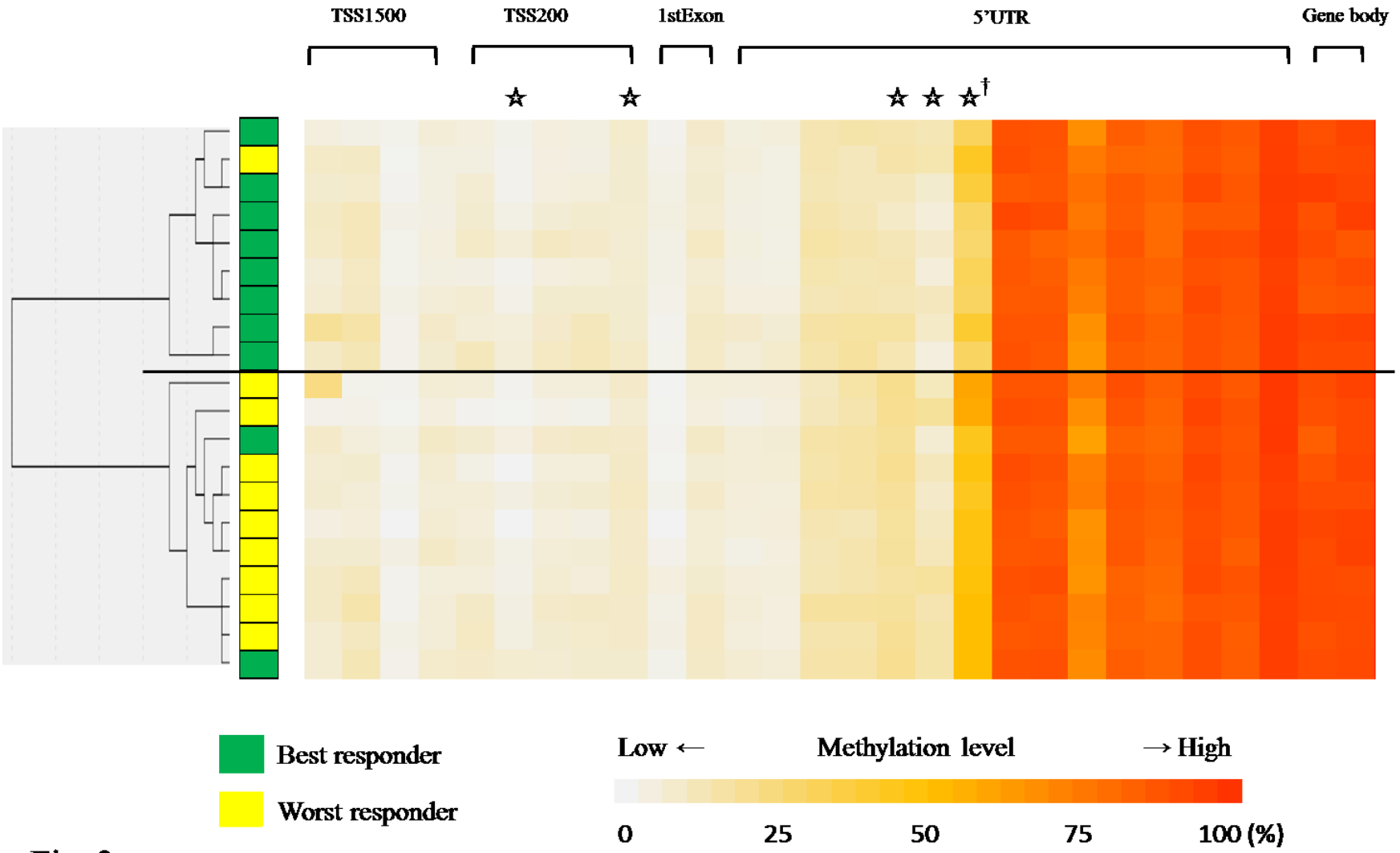
TSS1500
TSS200
1stExon
5'UTR
Gene body
Best responder
Worst responder
Low ←
Methylation level
→ High
0
25
50
75
100 (%)

Fig. 3.