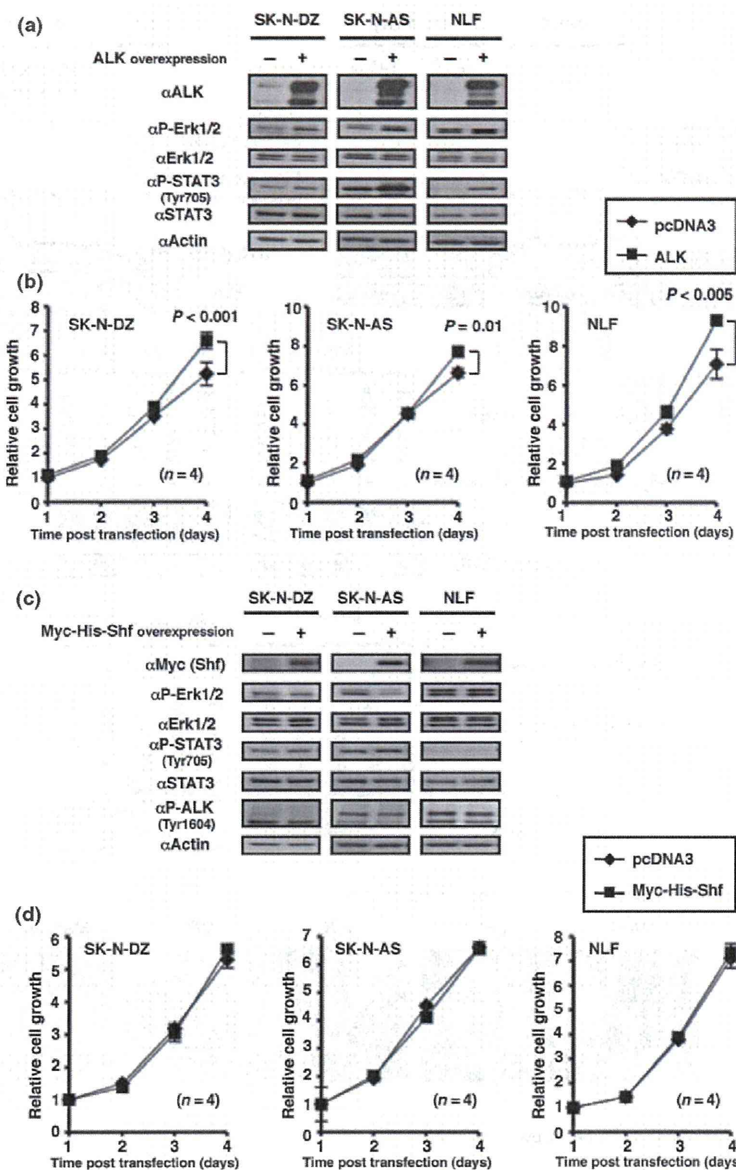

Fig. 3. Overexpression of anaplastic lymphoma kinase (ALK) facilitates cell growth and activates downstream signal pathways. (a) ALK overexpression induced phosphorylation of Erk1/2 and signal transducer and activator of transcription 3 (STAT3) in neuroblastoma cell lines SK-N-AS, SK-N-DZ, and NLF. (b) Cell growth promoted by exogenous expression of ALK. Growth rate was measured by WST assay. Mean values were calculated from quadruplicate experiments. Error bars show standard deviation. Contrarily, overexpression of Shf had least effect on the ALK signaling pathway (c) and cell growth (d).

ALK overexpression in NLF also yielded an increase of phosphorylation status of STAT3 at tyrosine 705, compared to individual treatment (Fig. 5d). These results suggest that Shf inhibits phosphorylation of ALK and STAT3, phopho-transduction signals that are downstream of ALK activation.[34,37,40] Therefore, we concluded that Shf negatively regulates phospho-transduction signals in ALK-oriented pathways, resulting in modulation of cell mobility and invasiveness in neuroblastoma.

Discussion

In this work, we identified that an adaptor protein Shf is a negative regulator of ALK and its downstream signals in neuroblastoma. High levels of *Shf* mRNA expression were observed in neuroblastomas with favorable outcome, whereas low expression was associated with unfavorable tumors. Shf interacts with ALK *in vivo*, suggesting the molecular function of Shf participating in ALK-oriented signal transduction pathways during neural development and tumorigenesis. In the absence of ALK, however, knockdown of *Shf* did not facilitate cell growth; overexpression of ALK stimulated the effect of *Shf* knockdown, suggesting that Shf inhibits the downstream signal initiated by ALK. Therefore, we concluded that the adaptor protein Shf interacts with ALK receptor and modulates oncogenic activity in neuroblastoma.

As an adaptor protein containing the SH2 domain, it can be implied that Shf may play multifunctional roles in a variety of aspects of cellular activity, depending on the interaction with different receptor proteins. Indeed, adaptor proteins bind to receptors at the cell membrane and regulate signal transduction pathways either positively or negatively. For instance, Shf suppresses a signal transduction initiated by PDGFα receptor, resulting in inhibition of apoptosis.[22] In contrast, Shb, another SH2-containing adaptor protein highly homologous to Shf, facilitates the PDGFα-oriented signal, leading to activation of apoptosis.[41] Structural differences between Shf and Shb may explain the molecular mechanism of this contradictory result.

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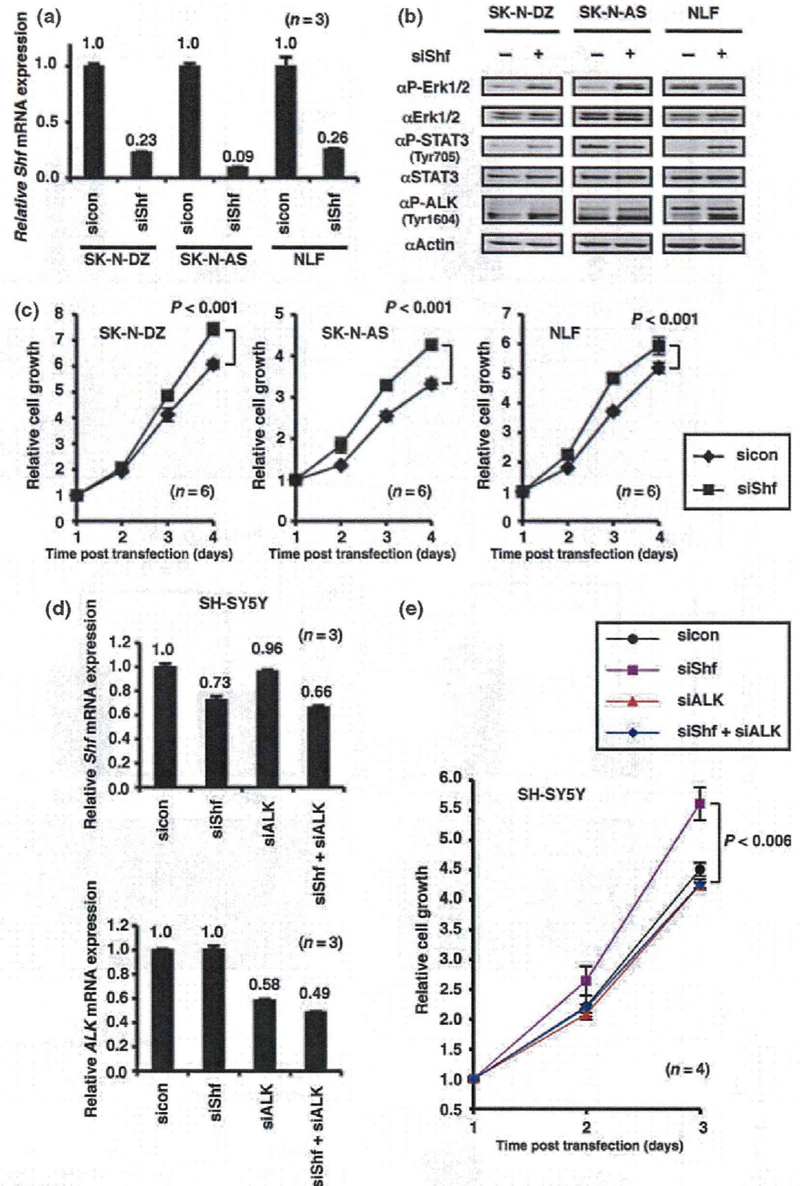

Fig. 4. Knockdown of *Shf* facilitates cell growth as well as activation of the anaplastic lymphoma kinase (ALK) pathway. (a) Knockdown of *Shf* mRNA mediated by specific siRNA was confirmed by real-time PCR. (b) *Shf* knockdown induced phosphorylation of ALK itself, Erk1/2, and signal transducer and activator of transcription 3 (STAT3) in neuroblastoma-derived cell lines. (c) Cell growth was facilitated when *Shf* was knocked down. (d) siRNA-mediated knockdown of *Shf* and *ALK*, confirmed by real-time PCR. The siRNA specific to *Shf* alone or those to *Shf* and *ALK* were used. (e) Growth effect of *Shf* knockdown in the presence or absence of *ALK*. Mean values of quadruplicate experiments are shown. sicon, siRNA control.

Compared to Shb, Shf lacks the PTB domain and proline-rich motifs at the N-termini, whereas the C-terminal region containing the SH2 domain is highly conserved (Fig. 1a). The SH2 domain is responsible for the binding to the receptor; the PTB domain is necessary to activate PDGFα. Therefore, Shf may act as a dominant negative competitor to Shb. In the case of ALK receptor tyrosine kinase, it has been well studied that ShcC, which is also a member of the SH2 adaptor protein family, facilitates the phospho-signal transduction initiated by ALK, inducing survival signals.[36,42] In this work, we showed that Shf negatively regulates the ALK signaling pathway, resulted in inhibition of cell growth and motility. This novel inhibitory mechanism mediated by Shf on the ALK signal pathway may confer the molecular model how adaptor proteins regulate phospho-transduction pathways that manage cell growth and mobility.[43–47]

This work showed that Shf physically binds to ALK and negatively regulates signal transduction downstream of the ALK pathway in neuroblastoma. Knockdown of *Shf* promoted phosphorylation of Erk/STAT accompanied by an increase in cell growth rate. Interestingly, this effect was nullified when ALK was simultaneously knocked down, indicating that existence of ALK is a prerequisite for suppression of ALK-oriented signal transduction mediated by Shf. This result suggested that Shf negatively regulates downstream of the ALK signal pathway. In addition, an increase of cell migration capability by *Shf* knockdown was significantly stimulated when ALK was exogenously overexpressed, further supporting the notion above. It should be noted that overexpression of ALK increased the growth of cells, but overexpression of Shf alone had no such effect (Fig. 3b, d). We speculate that this is due to the titration out of ALK protein by abundant Shf. This may also explain why Shf showed higher affinity with a constitutively active mutant (*F1174L*) of ALK than with wild-type (Fig. 2b). Abundant Shf protein may not be able to affect the mutant form of

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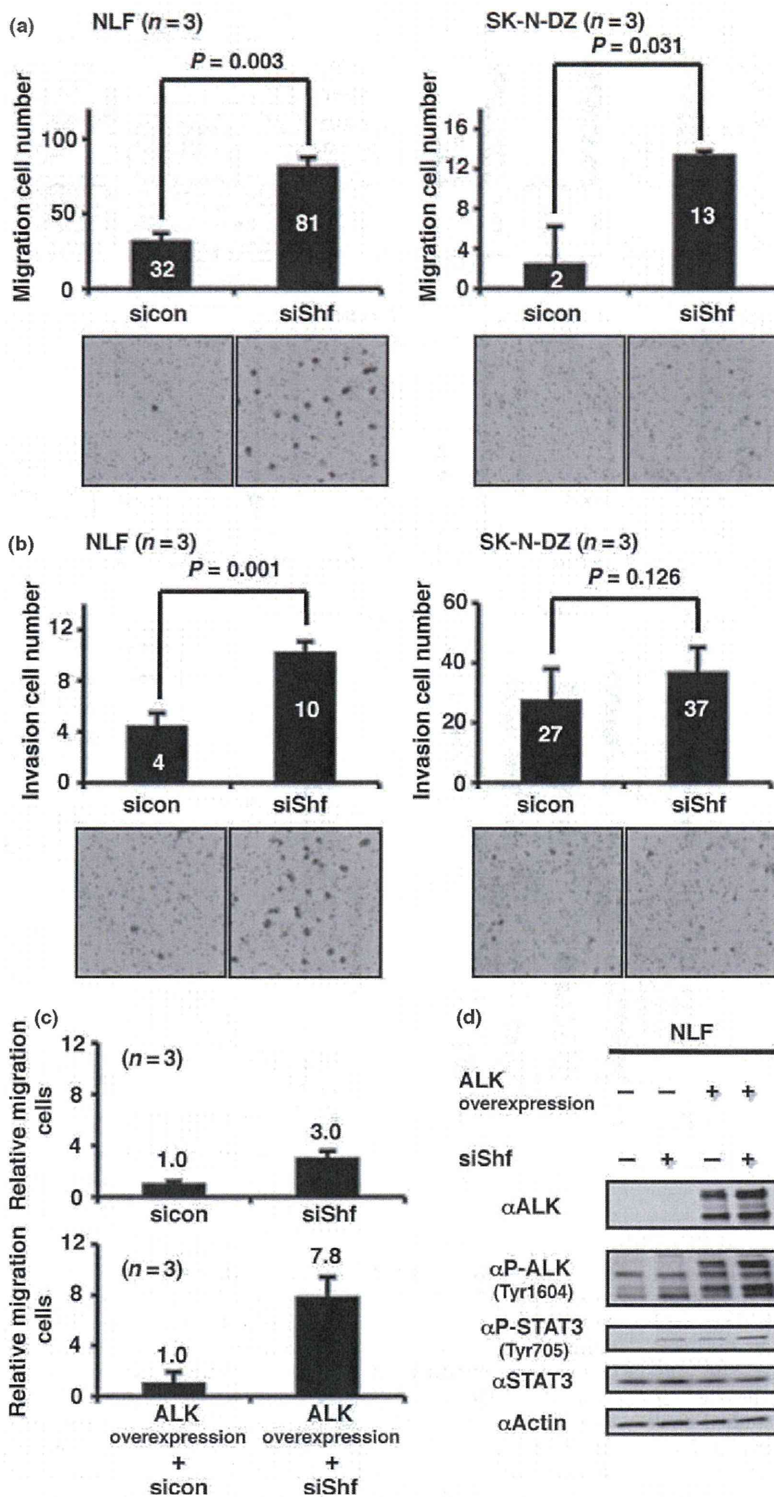

Fig. 5. Knockdown of *Shf* mediated by siRNA increases cellular motility and invasion capability in NLF and SK-N-DZ neuroblastoma cell lines. (a) Cellular migration was stimulated by knockdown of *Shf*. Mean values were calculated from independent triplicate experiments. Error bars indicate standard deviation. Representative blight field images are also shown. (b) Cellular invasion was promoted by *Shf* knockdown in neuroblastoma cell lines. (c) Cellular migration activity was stimulated by *Shf* knockdown. Cell migration assay was carried out in the presence or absence of expression vector of anaplastic lymphoma kinase (*ALK*). (d) *Shf* knockdown facilitated phosphorylation of ALK and signal transducer and activator of transcription 3 (STAT3) under the condition that ALK was overexpressed. sicon, siRNA control.

ALK, while the interaction between these two proteins was facilitated.

The ALK kinase inhibitor crizotinib (PF-02341066) reportedly inhibits proliferation of cells that express *R1275Q*-mutated ALK, whereas cells harboring *F1174L*-mutated ALK were relatively resistant.[48] In contrast, a small molecular weight compound TAE-684, another ALK inhibitor, decreased proliferation of human neuroblastoma cell lines harboring *F1174L*-mutated ALK.[15] Treatment of ALK^{F1174L} transgenic mice with TAE-684 induced complete tumor regression.[20] Therefore, combinations of the addback of Shf and the use of ALK inhibitors may be helpful to develop a potential treatment and cure for neuroblastoma.

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Disclosure Statement

The authors have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Relative Shf expression profiles in favorable/unfavorable samples.

Fig. S2. Tissue and cell line specificities of Shf and anaplastic lymphoma kinase (ALK).

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Stronger Prognostic Power of the CpG Island Methylator Phenotype than Methylation of Individual Genes in Neuroblastomas

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Objective: The CpG island methylator phenotype is strongly associated with poor survival in neuroblastomas. Neuroblastomas with the CpG island methylator phenotype include almost all neuroblastomas with *MYCN* amplification, and, even among neuroblastomas without *MYCN* amplification, have worse prognosis. At the same time, methylation of individual tumor-suppressor genes is also reported to be associated with poor survival. The purpose of this study was to compare the prognostic power of the CpG island methylator phenotype with that of methylation of individual genes.
Methods: Methylation-specific polymerase chain reaction was performed for five individual genes (*CASP8, EMP3, HOXA9, NR1I2* and *CD44*) in 140 Japanese and 152 German neuroblastomas. Kaplan–Meier analysis and log-rank tests were conducted to compare the survival between groups defined by methylation status.
Results: Among the five individual genes, only *CASP8* methylation had a significant association with poor overall survival both in Japanese (hazard ratio = 3.1; 95% confidence interval = 1.5–6.4; $P = 0.002$) and German (hazard ratio = 4.8; 95% confidence interval = 2.1–11; $P = 0.0002$) neuroblastomas. *HOXA9* and *NR1I2* methylation were associated with poor survival only in German neuroblastomas. On the other hand, the CpG island methylator phenotype had a strong and consistent association in Japanese (hazard ratio = 22; 95% confidence interval = 5.3–93; $P = 1.5 \times 10^{-5}$) and German (hazard ratio = 9.5; 95% confidence interval = 3.2–28; $P = 4.7 \times 10^{-5}$) neuroblastomas.
Conclusion: The CpG island methylator phenotype is likely to have stronger prognostic power than methylation of individual genes in neuroblastomas.

Key words: neuroblastoma – methylation – CIMP – poor survival

INTRODUCTION

Neuroblastoma (NBL) is the most frequent extracranial pediatric tumor (1). The CpG island methylator phenotype (CIMP), methylation of multiple CpG islands (CGIs), was associated with poor survival with a hazard ratio (HR) of 22 [95% confidence interval (95% CI) = 5.3–93] in Japanese and 9.5 (95% CI = 3.2–28) in German NBLs, respectively (2,3). The prognostic significance of CIMP was further confirmed in Italian NBLs by a pyrosequencing assay (4). Notably, NBLs with CIMP included almost all NBLs with *MYCN* amplification (37/38 in Japanese and 23/23 in German NBLs), the strongest current prognostic marker (5–7). Even among NBLs without

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MYCN amplification, CIMP was a significant and strong prognostic marker with an HR of 12 (95% CI = 2.6–59) in Japanese and 4.5 (95% CI = 1.3–16) in German NBLs.

CIMP is sensitively detected by methylation of marker CGIs, such as CGIs in gene bodies of the *PCDHB* gene family in NBLs. It is known that methylation of CGIs outside promoter regions (non-promoter CGIs) is not associated with loss of expression, and such non-promoter CGIs are more susceptible to methylation induction than promoter CGIs (8). As a model of the close association between methylation of non-promoter CGIs and poor survival, it was considered that CIMP consistently leads to methylation of non-promoter CGIs, such as CGIs of the *PCDHB* gene family in NBLs, and also to methylation of various promoter CGIs with low incidences, which causes poor survival.

At the same time, methylation of an individual gene has been also shown to be associated with poor survival. For example, methylation of *CASP8* was associated with poor survival with an HR of 5.3 (95% CI = 1.5–18; $P = 0.008$) (9). Methylation of *NR1I2*, *EMP3*, *HOXA9* and *CD44* was associated with poor survival with P values of 0.014, 0.03, 0.04 and 0.049, respectively (9–12). Functionally, *CASP8*, an apoptosis-related gene, has been reported to act as a tumor suppressor, and its loss is required for survival of NBL cells overexpressing *MYC* or *MYCN* (13). *NR1I2* (a nuclear receptor gene) and *EMP3* (a myelin-related gene) have been reported to have growth suppressive activity in NBL cells (10,11). However, the prognostic powers of methylation of these individual genes and of CIMP have never been analyzed in identical sets of NBLs.

In the present study, we aimed to compare the prognostic power of CIMP with that of methylation of individual genes.

PATIENTS AND METHODS

DNA Samples and Analysis of CIMP

The 140 Japanese and 152 German NBLs were identical with those analyzed in our previous studies (2,3). These samples were analyzed at the Division of Epigenomics, National Cancer Center Research Institute under the approval of institutional review boards. The presence of CIMP and *MYCN* amplification were determined as in our previous studies (2,3), and this information was used in the present study.

Sodium Bisulfite Modification and Methylation-specific Polymerase Chain Reaction (PCR)

Fully methylated DNA and fully unmethylated DNA were prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs, Beverly, MA, USA) and by amplifying genomic DNA with the GenomiPhi amplification system (GE Health Care, Buckinghamshire, UK), respectively. Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described (14), and the modified DNA was suspended in 40 μl of Tris–ethylenediaminetetraacetic acid buffer (pH 8.0). An aliquot of 1 μl was used for methylation-specific PCR (MSP).

MSP was performed using primers as previously published (11,13,15,16) (Supplementary data, Table S1). For the *NR1I2* gene, although the combined bisulfite restriction analysis was performed in the previous study (10), MSP targeting the same region was used in this study. Using fully methylated and unmethylated DNA, the annealing temperature that specifically amplified only methylated or unmethylated DNA was determined. Also, a minimum number of PCR cycles to obtain visible bands was determined using the (un)methylated DNA, and four cycles were added for the analysis of primary NBLs (Supplementary data, Table S1).

Statistical Analysis

Survival time was defined as the time between initial diagnosis and death, or time between diagnosis and last contact if no event had occurred. Kaplan–Meier analysis and log-rank tests were conducted to compare survival between the groups defined by methylation status. HRs were estimated by the Cox

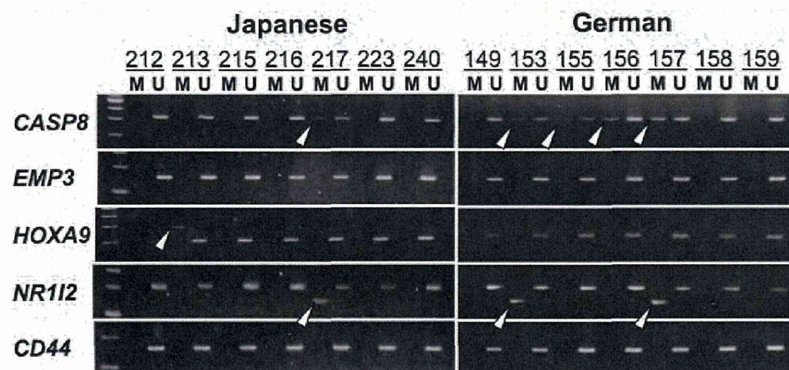

Figure 1. Methylation of promoter CpG islands (CGIs) of five individual genes (*CASP8*, *EMP3*, *HOXA9*, *NR1I2* and *CD44*) in Japanese and German neuroblastomas (NBLs). Representative results of methylation-specific PCR are shown. M and U, primers specific to methylated and unmethylated DNA, respectively. Arrowheads show the presence of methylated DNA molecules.

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proportional hazard model. These statistical analyses were performed using the SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Methylation of Individual Genes and their Prognostic Power Compared with CIMP

CASP8, *EMP3*, *HOXA9*, *NR1I2* and *CD44* were methylated in 26, 4, 27, 15 and 3, respectively, of the 140 Japanese NBLs, and in 30, 2, 2, 13 and 2, respectively, of the 152 German NBLs (representative results shown in Fig. 1). The prognostic power of methylation of the five genes was analyzed in Japanese and German NBLs, respectively (Fig. 2A and Table 1). In Japanese NBLs, only *CASP8* methylation had a significant association with poor survival (HR = 3.1; 95% CI = 1.5–6.4; $P = 0.002$). Regarding CIMP, defined by methylation of multiple genes and detected by methylation of the *PCDHB* gene family (2), it had a strong association with poor survival (HR = 22; 95% CI = 5.3–93; $P = 1.5 \times 10^{-5}$), and its prognostic power was stronger than that of *MYCN* amplification (HR = 9.5; 95% CI = 4.4–21; $P = 4.0 \times 10^{-9}$) (Fig. 2B). In the identical set of Japanese NBLs, a stronger prognostic power of CIMP than methylation of an individual gene was clearly shown.

In German NBLs, *CASP8* methylation was also associated with poor survival (HR = 4.8; 95% CI = 2.1–11; $P = 0.0002$) (Fig. 2A and Table 1). In addition, *HOXA9* and *NR1I2* methylation were associated with poor survival with an HR of 14 for *HOXA9* (95% CI = 3.1–62; $P = 0.0006$) and 4.2 for *NR1I2* (95% CI = 1.6–11; $P = 0.003$), respectively. Regarding CIMP and *MYCN*, as shown in our previous study (3), CIMP had a strong association with poor survival (HR = 9.5; 95% CI = 3.2–28; $P = 4.7 \times 10^{-5}$) and it was comparable to that of *MYCN* (HR = 12; 95% CI = 4.9–29; $P = 4.8 \times 10^{-8}$) (Fig. 2B). The stronger prognostic power of CIMP was consistently shown in the identical set of German NBLs.

Association Between CIMP and Methylation of Individual Genes

Among the five individual genes analyzed in this study, two genes (*CASP8* and *NR1I2*) were methylated at a significantly higher incidence in NBLs with CIMP (Fig. 3). In Japanese NBLs with and without CIMP, *CASP8* methylation was found in 24/67 and 2/73, respectively ($P = 5.0 \times 10^{-7}$). *NR1I2* methylation was found in 15/67 and 0/73, respectively ($P = 3.2 \times 10^{-5}$). Also in German NBLs with and without CIMP, *CASP8* methylation was found in 28/50 and 2/95, respectively ($P = 2.6 \times 10^{-14}$). *NR1I2* methylation was found in 11/50 and 1/95, respectively ($P = 1.4 \times 10^{-5}$). These results showed that CIMP was associated with methylation of multiple promoter CGIs, mainly *CASP8* and *NR1I2*.

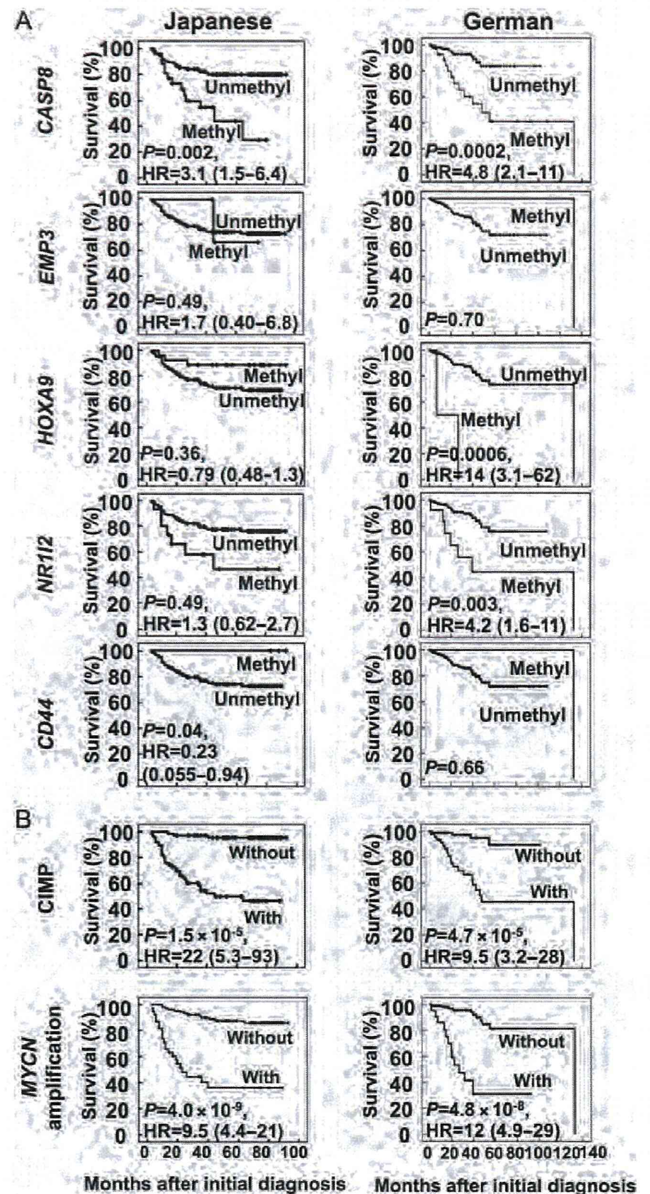

Figure 2. Prognostic power of (A) methylation of five individual genes (*CASP8*, *EMP3*, *HOXA9*, *NR1I2* and *CD44*), and (B) CpG island methylator phenotype (CIMP) and MYCN amplification in Japanese and German NBLs. Kaplan–Meier survival curves were drawn using the SPSS software. Among the five genes, only CASP8 methylation had a significant association with poor survival both in Japanese and German NBLs.

DISCUSSION

The stronger prognostic power of CIMP than methylation of individual genes was shown in this study. Also, the association between CIMP and methylation of multiple promoter CGIs was indicated. These results supported the idea that CIMP leads to a poor prognosis by induction of methylation of promoter CGIs of various tumor-suppressor genes with low incidences.

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Table 1. Prognostic power of methylation of individual genes and CpG island methylator phenotype (CIMP)

Marker	Japanese (n = 140)				German (n = 152)			
	No. of NBLs with methylation or amplification	HR	95% CI for HR	*P* value	No. of NBLs with methylation or amplification	HR	95% CI for HR	*P* value
CASP8	26	3.1	1.5–6.4	0.002	30	4.8	2.1–11	0.0002
EMP3	4	1.7	0.4–6.8	0.49	2	NA	–	0.70
HOXA9	27	0.79	0.48–1.3	0.36	2	14	3.1–62	0.0006
NR1I2	15	1.3	0.62–2.7	0.49	13	4.2	1.6–11	0.003
CD44	3	0.23	0.055–0.94	0.04	2	NA	–	0.66
CIMP	67	22	5.3–93	1.5×10^{-5}	50	9.5	3.2–28	4.7×10^{-5}
MYCN amplification	38	9.5	4.4–21	4.0×10^{-9}	23	12	4.9–29	4.8×10^{-8}

NBL, neuroblastoma; HR, hazard ratio; CI, confidence interval; NA, not applicable.

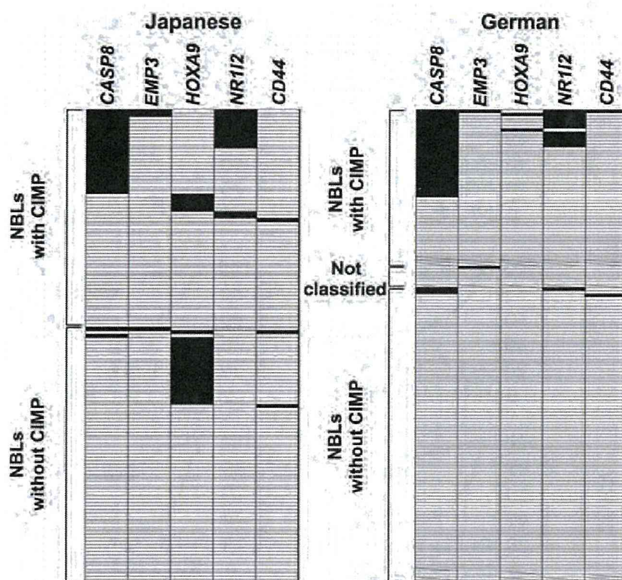

Figure 3. Methylation profiles of the five individual genes in NBLs with and without CIMP. Left panel, 140 Japanese NBLs; and right panel, 152 German NBLs. NBLs were classified by CIMP status determined as in our previous studies (2,3), and then aligned by methylation statuses of the five genes. In German NBLs, seven cases were not classified as NBLs with CIMP or without CIMP (3). The NBLs with CIMP tended to show methylation of multiple promoter CGIs. Closed box, methylated DNA detected; open box, only unmethylated DNA detected; and box with a slash; neither methylated nor unmethylated DNA detected, possibly due to low DNA quality.

Regarding the assessment of CIMP, besides the use of the *PCDHB* gene family, a combination of silenced genes has been proposed. Yang et al. (17) analyzed methylation of eight genes (*HIC-1, RASSF1A, BLU, DCR2, CASP8, TIG-1, HIN-1, TMS-1*), and identified that methylation of two and three genes had no effects on survival ($P = 0.719$ and 0.214, respectively), but methylation of ≥ 4 genes had a trend toward decreased survival ($P = 0.055$). Also, Lau et al. (18) identified that methylation of at least one of three genes (*FOLH1, MYOD1* and *THBS1*) was associated with event-free survival (HR = 2.2; 95% CI = 1.1–4.2; $P = 0.022$), and the association was stronger in methylation of all the three genes (HR = 4.5; 95% CI = 1.6–13; $P = 0.006$). These data support the model that CIMP leads to methylation of promoter CGIs of tumor-related genes with low incidences, which leads to poor survival.

Among the individual genes, *CASP8* and *RASSF1A* methylation have been repeatedly shown to be associated with poor survival (9,17,19–23). *CASP8* methylation was consistently associated with poor survival in the present study. By the analysis of methylation and survival data in our previous study (2), *RASSF1A* methylation was also revealed to be associated with poor survival in Japanese NBLs (HR = 4.2; 95% CI = 1.9–9.3; $P = 0.0005$). However, HRs of these genes were smaller than that of CIMP. These data indicated that these two genes play critical roles in a fraction of NBLs but not in the other NBLs. Indeed, a recent genome-wide methylation study revealed that methylation of numerous genes was associated with poor survival in NBLs (24).

In conclusion, the stronger prognostic power of CIMP than of methylation of individual genes was shown, and methylation silencing of various tumor-suppressor genes with low incidences was suggested to be involved in poor survival.

Supplementary data

Supplementary data are available at http://www.jjco.oxfordjournals.org.

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Conflict of interest statement

None declared.

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