厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

腫瘍内低酸素ががんの進展とがん幹細胞の生存維持に及ぼす影響

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研究要旨　ルイス肺癌由来の高転移性細胞 A11 で見出したミトコンドリア（mt）*ND6* 遺伝子中のミスセンス変異（G13997A）が呼吸鎖 complex I の活性低下と活性酸素種（ROS）の高産生を介して、低酸素誘導アポトーシス抵抗性と VEGF 高産生能を賦与し、転移能の制御に係るという昨年度の知見を、他の細胞系およびヒト肺癌の転移性脳腫瘍で検討した。まず、非転移性 B82 線維肉腫細胞の mtDNA を A11 細胞の mtDNA と完全に入れ替えたサイブリッド（B82mtA11 細胞）を樹立し ROS 産生能と転移能を調べた。その結果、対照のサイブリッド（B82mtB82 細胞）と比較して、complex I 活性の低下と ROS の産生が促進されるとともに、転移能が亢進されていることが判明した。従って、A11 細胞の *ND6* 遺伝子中のミスセンス変異が B82 細胞にも転移性を賦与することが判った。また、B82 細胞由来の高転移性細胞 B82Met では、complex I 活性の低下と ROS の高産生が起きており、*ND6* 遺伝子中にナンセンス変異が存在することが明らかになった。次に、ヒト転移性脳腫瘍における *ND6* 遺伝子の変異を調べたところ、27 症例中の 3 例において 4 種類のミスセンス変異が見出された。

A. 研究目的

ルイス肺癌由来の高転移性細胞 A11 は、低転移性細胞 P29 と比較して、(a) 抗アポトーシス蛋白質 Mcl-1 を高発現するため低酸素やグルコース欠乏によるアポトーシス誘導に対して抵抗性を示し、(b) 低酸素下で HIF-1α及び VEGF を高発現するため血管新生能が高く、(c)活性酸素種(ROS)を多量に産生する。昨年度、ROS の産生とミトコンドリアとの関連及び ROS の産生と転移能との関連を、細胞間でミトコンドリア DNA（mtDNA）を完全に交換したサイブリッド細胞を用いて検討し（筑波大学・林純一教授との共同研究）、*ND6* 遺伝子中のミスセンス変異（G13997A）が呼吸鎖 complex I の活性の低下と ROS の高産生を惹起し、これが原因で Mcl-1 と HIF-1α の高発現をもたらし、転移能を亢進させることを示した。そこで、この *ND6* 遺伝子中のミスセンス変異が他の低転移性細胞株においても転移能を亢進させる作用があるのかどうか、またヒトがんの転移巣においても *ND6* 遺伝子中にミスセンス変異があるのかどうかを検討した。

B. 研究方法

細胞は、非転移性マウス線維肉腫 B82 および B82 細胞由来の高転移性 B82Met 細胞、ルイス肺癌由来の低転移性 P29 細胞及び高転移性 A11 細胞を用いた。サイブリッド細胞は、B82 細胞から樹立した mtDNA-less 細胞（ρ^0B82）と脱核した A11 細胞と細胞融合させて作製した B82mtA11 細胞（核 DNA は B82 由来で、mtDNA は A11 由来）を用いた。対象として B82mtB82（核 DNA、mtDNA 共に B82 由来）を用いた。Complex I+III 活性は NADH と cytochrome c (oxidized form)を基質として用いた比色法で、ROS 産生は DCFH-DA 染色後に flow cytometry で、mtDNA 遺伝子型は PCR-RFLP 法で、転移能はヌードマウスの尾静脈内移植で検討した。

（倫理面への配慮）

ヒト転移性脳腫瘍におけるミトコンドリア DNA の変異解析は、千葉県がんセンター倫理審査委員会の承認後に行なった。

C. 研究結果

B82mtA11 細胞を樹立し、mtDNA が完全に交換されていることを PCR-RFLP 法で確認した。そこで、B82mtA11 細胞と対照として樹立した B82mtB82 の転移能を調べたところ、B82mtA11 細胞が高転移性を示すことが判った。さらに、B82 細胞由来の高転移性細胞 B82Met 細胞の mtDNA 中の変異を調べたところ、*ND6* 遺伝子中にナンセンス変異が存在することが明らかになった。これらの *ND6* 遺伝子の変異を有する細胞では、呼吸鎖 complex I 活性の低下による ROS の高産生が認められた。さらに、ヒト肺癌の転移性脳腫瘍 27 例における *ND6* 遺伝子

の変異を調べたところ、3 症例において 4 種類のミスセンス変異が検出された。

D. 考察

A11 細胞の *ND6* 遺伝子中の病因性ミスセンス変異が細胞種の異なる B82 細胞の転移性を亢進させるという実験結果は、呼吸鎖活性の低下および ROS の発生につながる mtDNA 中の変異が転移能を制御するという概念にある程度の一般性を与えるものと考えられる。高転移性の B82Met 細胞において *ND6* 遺伝子中にナンセンス変異が見いだされたこともこの考えを支持するものと思われる。肺がんの脳転移巣 27 症例中 3 例において *ND6* 遺伝子中に 4 種類のミスセンス変異が見いだされたが、現在のところこれらの変異が病因性であるという明確な証拠はないためにその意義は不明である。将来の研究において明らかにされるべき点であろう。

E. 結論

Complex I 活性を低下させ ROS の高産生の原因となる mtDNA の病因性変異により転移能が制御されるという新しい転移制御の概念が示唆された。

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H. 知的財産権の出願・登録状況

1． 特許取得

なし

2． 実用新案登録

なし

3． その他

なし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

翻訳調節を介した細胞増殖と老化の総合的制御メカニズムの解明

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研究要旨
ほ乳類ポリコム群は、Ink4a/p53経路に対して抑制的に作用して細胞老化を制御することが遺伝学的に示されている。この抑制は、ポリコム群複合体のInk4a遺伝子座への直接結合を介していることを今まで示してきた。昨年度までに、新規ポリコム群タンパクPcl２はポリコム群に対し拮抗的に作用し、その作用機序は翻訳調節を介したポリコム群タンパクの発現制御メカニズムであることを新たに示し、新たながん抑制候補遺伝子であることを示した。Pcl２の機能発現機序を明らかにするために、本年度は、そのTudorドメイン及びふたつのPHDフィンガーについて構造解析及び生化学的特性の解析を行った。その結果、いずれも異なるヒストン修飾を認識しうることが示され、Pcl２はヒストンコードを文脈として読み取るタンパクであることが示唆された。また、ヒストン以外のメチル化タンパクを認識しうる可能性が示唆された。

A．研究目的

ほ乳類ポリコム群は、Ink4a/p53 経路に対して抑制的に作用して細胞老化を制御することが遺伝学的に示されている。この抑制は、ポリコム群複合体の Ink4a 遺伝子座への直接結合を介していることを今まで示してきた。昨年度までに、新規ポリコム群タンパク Pcl２はポリコム群に対し拮抗的に作用し、その作用機序は翻訳調節を介したポリコム群タンパクの発現制御メカニズムであることを新たに示し、新たながん抑制候補遺伝子であることを示した。Pcl２の機能発現機序を明らかにするために、本年度は、その Tudor ドメイン及びふたつの PHD フィンガーについて構造解析及び生化学的特性の解析を行った。

B．研究方法

TudorドメインとPHD１およびPHD２について、組み換えタンパクを麦芽抽出物を用いて大量発現させた上で精製した。可溶性を確認した上で、NMRを用いた溶液中での立体構造解析を試みた。リガンドについては、構造から予測されたリガンド（ヒストンテール）を各種合成し、ビアコア、あるいはNMRにより結合の有無を解析した。PHD２については、十分な可溶性が得られなかったため、通常のプルダウン法を用いてリガンドの決定を行った。

（倫理面への配慮）

遺伝子組換え実験と動物実験については、以下に示す文部科学省及び環境省関係法令・指針に準拠して定められた理研所内規程に則って行っている。

１．遺伝子組換え実験

【関係法令・指針】

遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律

【理研所内規程】

横浜研究所遺伝子組換え実験実施安全管理規程

横浜研究所遺伝子組換え実験に関わる申請及び承認に関する細則

C．研究結果

Tudor ドメインが構成するポケットは、内部に突出したトリプトファンの向きにより、二通りの構造を取りうることが示された。複数のリガンドの存在が想定され、トリメチル化されたヒストンＨ３リシン（K）４、９、２７のいずれにも結合し得ただけでなく、二本鎖 DNA や RNA も認識することが示された。一方、PHD１についても構造を決定し、そのポケットの構造を明らかにした。PHD１は、メチル化されていないＨ３Ｋ４を認識することが示された。PHD２は、構造解析をするのに十分な可溶性のあるポリペプチドは調整できず構造を明らかにすることはできなかった。しかしな

がら、通常のプルダウン法により、トリメチル化されたH3K4に結合しうることが明らかになった。

D．考察

Tudor ドメイン及びふたつの PHD フィンガーについて構造解析及び生化学的特性の解析を行った。その結果、いずれも異なるヒストン修飾を認識しうることが示され、Pcl2はヒストンコードを文脈として読み取るタンパクであることが示唆された。また、ヒストン以外のメチル化タンパクを認識しうる可能性が示唆された。

E．結論

Pcl2はポリコム群が作用するにあたって、クロマチン状況を読み取って、そのポリコム群の機能発現を制御するためのモジュールであることが示唆された。今後、がん抑制遺伝子として作用する際には、何を認識しているのかを明らかにしていく必要がある。

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H．知的財産権の出願・登録状況
（予定を含む。）
なし。

Ⅲ．研究成果の刊行に関する一覧表

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Marked and independent prognostic significance of the CpG island methylator phenotype in neuroblastomas ☆

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Abstract

The CpG island methylator phenotype (CIMP) was closely associated with poor overall survival (OS) in Japanese neuroblastoma (NBL) cases in our previous study. Here, in German NBL cases, CIMP(+) cases ($n = 95$) showed markedly poorer OS (hazard ratio (HR) = 9.5; $P < 0.0001$) and disease-free survival (DFS) (HR = 5.4; $P < 0.0001$) than CIMP(−) cases ($n = 50$). All the 23 cases with N-*myc* amplification had CIMP. Among the remaining cases without N-*myc* amplification, CIMP(+) cases ($n = 27$) had a poorer OS (HR = 4.5; $P = 0.02$) and DFS (HR = 5.2; $P < 0.0001$) than CIMP(−) cases ($n = 95$). In multivariate analysis, CIMP and N-*myc* amplification had an influence on OS and DFS independent of age and disease stage. CIMP had a stronger influence on DFS than N-*myc* amplification while N-*myc* had a stronger influence on OS.

Keywords: Neuroblastoma; Methylation; CIMP; MS-RDA; N-*myc*

1. Introduction

Neuroblastoma (NBL) is one of the most common pediatric solid tumors, and is characterized by two extreme disease courses, spontaneous regression and life-threatening progression. To implement adequate and necessary therapeutics, NBL cases are stratified into low-, intermediate- and high-risk groups based upon clinical and genetic information, such as disease stage, age at diagnosis, Shimada histology, N-*myc* amplification status, DNA ploidy, and *TrkA* expression level [1–6]. Especially, N-*myc* amplification, present in approximately 20–30% of NBL cases, is a powerful molecular marker for the stratification [1–4]. Nevertheless, more precise risk estimation is necessary for cases currently stratified

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into the intermediate-risk group, and development of a novel prognostic marker is awaited [1,2].

Recently, using a genome-wide screening method for differences in DNA methylation, methylation-sensitive representational difference analysis [7–9], we found that multiple CGIs were methylated in NBL cases with poor prognosis [10]. By analysis of 140 Japanese NBL cases, methylation of the multiple CGIs was shown to be dependent upon each other, and conformed to the concept of the CGI methylator phenotype (CIMP), originally established in colorectal cancers [11]. Cases could be classified as either CIMP(+) or CIMP(−), and a very limited number of cases had an intermediate phenotype. CIMP(+) cases had a markedly poorer overall survival (OS) than CIMP(−) cases with a hazard ratio (HR) of 22.1 [95% confidence interval (95%CI) = 5.3–93.4; $P < 0.0001$]. Its influence was independent of *TrkA* expression status, DNA ploidy, and age at diagnosis. Notably, almost all cases with N-*myc* amplification exhibited CIMP (37 of 38 cases), and, even among the cases without N-*myc* amplification, CIMP(+) cases had a poorer OS than CIMP(−) cases (HR = 12.4; 95%CI = 2.6–58.9; $P = 0.002$). CIMP status was well associated with the methylation level of the *Protocadherin β* (*PCDHB*) gene family, followed by methylation levels of *hepatocyte growth factor-like protein* (*HLP*) gene and *Cytochrome p450 CYP26C1* (*CYP26C1*).

Considering that there could be potential ethnic differences and that genome-wide screenings tend to produce "too good" results [12], here we took advantage of archived materials of German NBL cases. If the strong influence of CIMP on OS is also observed in German cases, we can establish CIMP as a prognostic marker that can be universally used. Also, the German NBL cases have information on disease-free survival (DFS), which was not available for Japanese NBL cases, and the influence of CIMP on DFS can be clarified.

2. Materials and methods

2.1. Tissue samples

A total of 152 cases were collected between 1998 and 2004, and all patients were enrolled in the German NBL Trial. The mean age at initial diagnosis was 1082 days (range 0–9607 days). Thirty-seven, 29, 17, 51 and 17 cases belonged to stages 1, 2, 3, 4, and 4S (International Neuroblastoma Staging System), respectively, although information was not available for one case. The composition of the cohort in terms of stage, N-*myc* status and age at diagnosis was in agreement with the composition of an unselected cohort of 1741 patients diagnosed between 1990 and 2003 in Germany [13]. DNA was extracted by the standard phenol/chloroform procedure, and used for this study under approval of Institutional Review Boards.

2.2. Sodium bisulfite modification and quantitative methylation-specific PCR (MSP)

One microgram of DNA restricted with *Bam*HI underwent sodium bisulfite modification [14], and was suspended in 20 μl of TE buffer. For quantitative MSP, 1 μl of the solution was used for PCR using SYBR Green PCR Core Reagents (PE Biosystems) and an iCycler Thermal Cycler (Bio-Rad Laboratories). PCR was performed separately for methylated (M) DNA molecules and for unmethylated (U) DNA molecules with primers specific to each sequence, and the numbers of M and U molecules in a test sample were determined by comparing their amplification with those of standard samples containing $10–10^6$ molecules. Primer sequences and standard DNA were previously described [10]. The "methylation level" was calculated as the fraction of M molecules in the total DNA molecules (# of M molecules + # of U molecules). All the molecular analyses were performed blind to clinical information, and methylation level for a case was obtained as an average of two independent measurements.

2.3. Statistical analysis

Reproducibility of methylation levels between two measurements was assessed using the Pearson correlation coefficient. Survival time was measured from the date of initial diagnosis to the date of death or last contact. Kaplan–Meier analysis and log-rank tests were performed to compare overall survival (OS) and disease-free survival (DFS) between groups. HRs were estimated by the Cox proportional hazards model. These statistical analyses were performed using SPSS, version 13.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Determination of CIMP statuses in German NBL cases

Methylation levels were measured in 152 German NBLs for three CGI (group)s – (i) the 17 *PCDHB* family genes, (ii) *HLP*, and (iii) *CYP26C1*. They were highly reproducible with a correlation coefficient $\geqslant 0.99$, and the average levels were used hereafter. The methylation level of the *PCDHB* gene family showed a clear bimodal distribution (Fig. 1A). To avoid artificial bias, CIMP statuses were diagnosed before having access to clinical information of the cases. First, since cut-off values between 40% and 60% gave high HRs in our previous

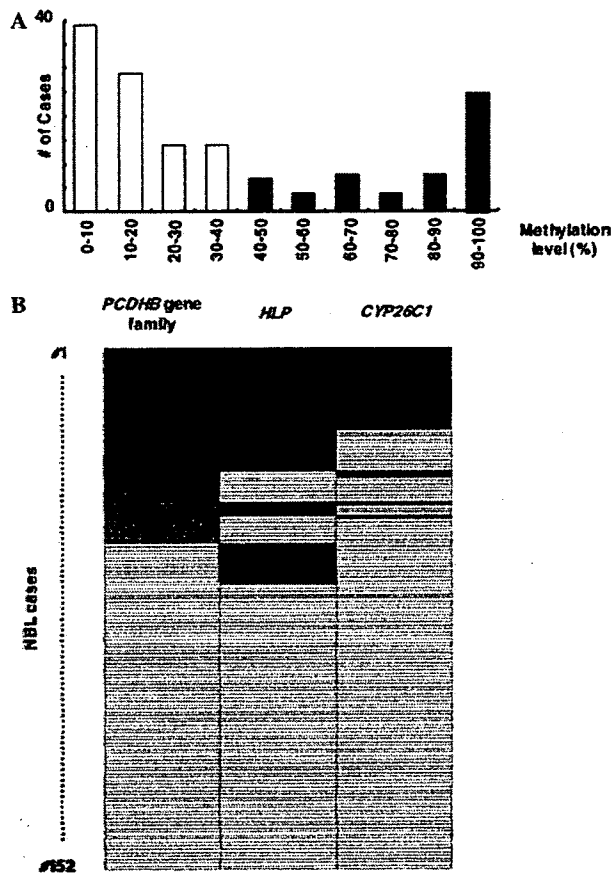

Fig. 1. Bimodal distribution of methylation levels of the *PCDHB* gene family, and diagnosis of CIMP status. (A) Histogram of number of cases according to *PCDHB* methylation levels. The methylation level of the *PCDHB* gene family was measured exactly as in our previous study [10], and its bimodal distribution in German NBLs was confirmed. (B) Methylation statuses of the three CGIs (groups) among the 152 NBLs. Cut-off values for the *PCDHB* gene family, *HLP* and *CYP26C1* were set based on the previous study, which were 40–60%, 10%, and 70%, respectively. Closed and open boxes show high and low methylation levels, and methylation levels of the *PCDHB* gene family between 40% and 60% are shown by grey boxes. Methylation levels of these three CGIs were closely associated with each other.

study [10], cases with methylation levels lower than 40% and higher than 60% were diagnosed as CIMP(−) ($n = 95$) and CIMP(+) ($n = 45$), respectively. Only 12 cases had methylation levels between 40% and 60%.

Then, for these 12 cases, methylation levels of *HLP* and *CYP26C1*, whose predictive powers followed that of the *PCDHB* gene family in our previous study [10], were taken into account. Five of the 12 cases had high levels of methylation of *HLP* and/or *CYP26C1*, and were considered to have CIMP, and seven other cases were left as unknown for CIMP status (Fig. 1B). Cut-off values for *HLP* and *CYP26C1* were set at the same levels as in our previous study, which were 10% and 70%, respectively. As a result, 50, 95, and 7 cases of the 152 cases were diagnosed as CIMP(+), CIMP(−), and unknown, respectively. Methylation statuses of the three CGI (groups) showed close correlation with methylation statuses of the other CGIs.

3.2. Univariate analysis with OS and DFS

In univariate analysis, the 50 CIMP(+) cases exhibited markedly and significantly poorer OS (HR = 9.5; 95%CI = 3.2–28.1; $P < 0.0001$) and DFS (HR = 5.4; 95%CI = 2.9–10.3; $P < 0.0001$) than the 95 CIMP(−) cases. Cases with N-*myc* amplification ($n = 23$) also exhibited markedly and significantly poorer OS (HR = 11.8; 95%CI = 4.9–28.7; $P < 0.0001$) and DFS (HR = 3.1; 95%CI = 1.6–6.0; $P = 0.0007$) than 122 cases without N-*myc* amplification. All of the 23 German cases with N-*myc* amplification had CIMP, as observed in a Japanese population.

Therefore, the German NBL cases were classified into three groups: (a) CIMP(−) cases ($n = 95$), all of which were without N-*myc* amplification, (b) CIMP(+) cases without N-*myc* amplification ($n = 27$), and (c) CIMP(+) cases with N-*myc* amplification ($n = 23$). As for OS (Fig. 2A), the three groups exhibited a step-wise increase of risk, showing the influence of N-*myc* amplification in addition to CIMP. Among the cases without N-*myc* amplification (groups (a) and (b)), CIMP had a significant influence on OS (HR = 4.5; 95%CI = 1.3–16.1; $P = 0.02$). As for DFS (Fig. 2B), CIMP had a significant influence (HR = 5.2; 95%CI = 2.6–10.6; $P < 0.0001$) by comparison of groups (a) and (b). However, additional influence by N-*myc* amplification was unclear by comparison of groups (b) and (c). These suggested that N-*myc* amplification had a strong influence on OS while CIMP had a strong influence on DFS.

3.3. Multivariate analysis

Since CIMP and N-*myc* amplification were dependent upon each other, multivariate analysis was first performed using age at diagnosis, disease stage, and either CIMP or N-*myc* amplification (Table 1A and B). It was confirmed that either CIMP or N-*myc* amplification had a significant influence on OS and DFS independent of age at diagnosis and disease stage.

Then, multivariate analysis was performed using age at diagnosis, disease stage, and both CIMP and N-*myc* amplification to compare the influences of them (Table 1C). As for OS, N-*myc* amplification retained its power while CIMP lost its power. In contrast, as for DFS, CIMP retained its power while N-*myc* amplification lost its power. This result was in accordance with the finding that CIMP had a strong influence on DFS while N-*myc* amplification had a strong influence on OS.

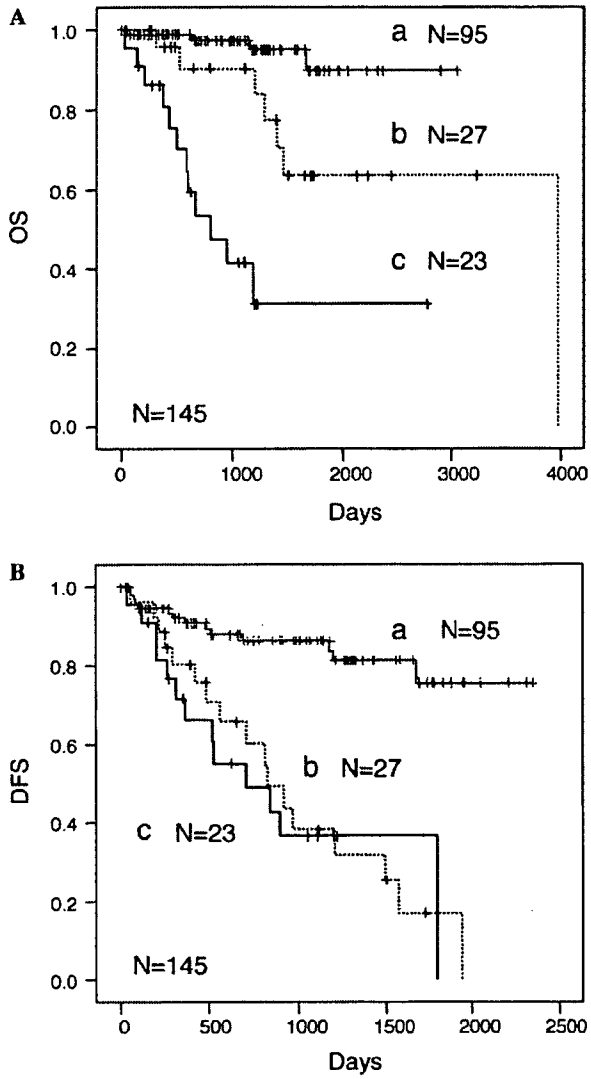

Fig. 2. Kaplan–Meier analysis of (a) CIMP(−) cases without N-*myc* amplification ($n = 95$), (b) CIMP(+) cases without N-*myc* amplification ($n = 27$), and (c) CIMP(+) cases with N-*myc* amplification ($n = 23$). (A) Kaplan–Meier analysis using OS. Using group (a) as a reference, group (b) had a HR of 4.5 (95%CI = 1.3–16.1; and $P = 0.02$), and group (c) had a HR of 21.7 (6.8–69.3; <0.0001). Using group (b) as a reference, group (c) had a HR of 4.8 (1.7–13.6; 0.003). (B) Kaplan–Meier analysis using DFS. Using group (a) as a reference, group (b) had a HR of 5.2 (2.6–10.6; <0.0001), and group (c) had a HR of 5.7 (2.6–12.2; <0.0001). There was no significant difference between groups (b) and (c) ($P = 0.82$).

4. Discussion

Methylation levels of the *PCDHB* gene family showed a bimodal distribution in German NBL cases, as in our initial analysis of Japanese NBL cases [10], and the presence of two groups of NBLs from the viewpoint of CIMP was confirmed. The CIMP statuses of individual German NBL cases were determined using criteria established in Japanese NBL cases to avoid falsely "too good" results, which tend to happen in genome-wide analyses [12]. Nevertheless, the strong influence of CIMP on OS in all the NBL cases (HR = 9.5) and also in those without N-*myc* amplification (HR = 4.5) was confirmed. After finishing all the analysis we searched for a *PCDHB* methylation level that would give the highest HR for the 152 German NBL cases, and it was 30% with a HR of 9.8 (95%CI = 2.9–33.0; $P < 0.0001$), followed by 40% with a HR of 9.4 (95%CI = 3.2–27.6; $P < 0.0001$). Based on the precise reproduction of the initial findings in Japanese NBL cases in German NBL cases, CIMP is highly likely to be a novel prognostic marker that can be universally used in cases without N-*myc* amplification. A prospective study is warranted.

A strong influence of CIMP on DFS was revealed for the first time in this study because data on DFS were available only for German NBL cases. In univariate analysis, CIMP had a strong influence on DFS in all the NBL cases (HR = 5.4) and in the cases without N-*myc* amplification (HR = 5.2) (groups (a) and (b) in Fig. 2B). In multivariate analysis involving age at diagnosis, disease stage, and both N-*myc* amplification and CIMP, CIMP retained its power on DFS while N-*myc* amplification retained its power on OS. This suggested that the recurrence of NBL cases was strongly associated with CIMP, but that NBL cases without N-*myc* amplification had higher chances to be induced into the second remission.

The almost complete inclusion of cases with N-*myc* amplification within the CIMP(+) cases in our two independent studies indicates that these two abnormalities are very closely associated with each other. If we assume a single abnormality that underlies a poor prognosis of NBL cases, it is likely that CIMP is caused by it, and some of CIMP(+) NBLs develop N-*myc* amplification. If we assume multiple abnormalities, it is likely that CIMP is consistently associated with the devastating status of NBLs, which can be induced by N-*myc* amplification and other causes. Clarification of what molecular abnormality causes CIMP and how CIMP and N-*myc* amplification are related is important.

The presence of CIMP was considered to lead to a poor prognosis by induction of methylation of promoter CGIs of various tumor-related genes. We

Table 1
Multivariate analysis of prognostic factors for overall and disease-free survival

Variable	OS			DFS		
	HR	95% CI for HR	*P*	HR	95% CI for HR	*P*
(A)						
Age at diagnosis	6.2	0.8–48.7	0.082	1.8	0.8–4.1	0.171
Disease stage	1.8	0.6–5.8	0.319	1.8	0.8–4.0	0.152
CIMP	4.9	1.5–15.8	0.008	3.3	1.5–7.0	0.002
(B)						
Age at diagnosis	13.6	1.8–104.3	0.012	2.5	1.1–5.6	0.025
Disease stage	1.5	0.5–5.0	0.501	2.6	1.2–5.4	0.013
N-*myc* amplification	11.5	3.9–33.8	<0.001	2.1	1.0–4.2	0.043
(C)						
Age at diagnosis	12.1	1.6–94.4	0.017	1.9	0.8–4.5	0.137
Disease stage	1.2	0.3–4.1	0.796	1.7	0.8–3.9	0.179
N-*myc* amplification	8.0	2.5–25.8	<0.001	1.3	0.6–2.7	0.563
CIMP	2.3	0.6–8.9	0.226	3.0	1.3–6.9	0.009

HR, hazard ratio; CI, confidence interval; OS, overall survival; DFS, disease-free survival.

previously observed association between CIMP and promoter methylation of tumor-suppressor *RASSF1A* and *BLU* genes [10]. It is reported that an anti-apoptotic gene, *TMS1*, a homeobox gene, *HOXA9*, a cell cycle gene, *CCND2*, and candidate tumor-suppressor genes, *EMP3* and *NR1I2*, are more frequently methylated in NBL cases with a poor prognosis [15–17]. However, the risk given by methylation of one of these individual genes is much smaller than that given by CIMP. This is in accordance with our hypothesis that CIMP leads to consistent methylation of marker CGIs, such as exonic CGIs of the *PCDHB* gene family, and occasional methylation of promoter CGIs of tumor-related genes. Silencing of an individual gene accounts for a poor prognosis of only a fraction of NBL cases with CIMP. It is known that exonic CGIs are more susceptible to methylation than promoter CGIs [9], and it is expected that they are more useful as a prognostic marker.

In summary, the faithful reproduction in German NBL cases of the highly significant findings obtained in Japanese cases demonstrated that CIMP is a strong and universal prognostic marker for NBL cases, especially for those without N-*myc* amplification. The close association between CIMP and DFS was revealed for the first time in this study.

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