時の DNA と健常人ボランティア 8 人から抽出した Buffy coat DNA を用いて作成したその希釈系列（10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}）とともに，各再構成に対して設定されたプライマー[9,13]と ASO プライマーを用いて nested PCR[13,15]を行った．各 PCR 産物をエチジウムブロマイドにて染色した 2%アガロースゲルで電気泳動し，希釈系列のバンドの信号強度と比較して MRD の定量を行い，10^{-4} 以上を陽性，それ未満を陰性とした．

なお，検体の採取，使用にあたっては患者家族の同意を得たうえで行った．

III. 結　　果

再発時骨髄検体の遺伝子再構成スクリーニングで同定しえた各症例の再構成と特異的塩基配列およびこれを用いて設計した ASO プライマーを Table 3 に示す．再構成は 2 例が TCR γ 遺伝子，2 例が Ig κ 遺伝子に生じており，症例 1 のみが初発時と異なるクローンであった．

各症例の再発後の経過・転帰と MRD 定量の結果を Fig. 2 に示す．症例 1 と 2（S2 群）は ALL-REZ BFM2002 研究[16]に基づいた化学療法を開始し，症例 1 は形態学的には再寛解が得られたが，Point 1 における MRD 量が 10^{-2} のレベルで陽性であった（Fig. 3）．このため大量シタラビンを中心とする化学療法で治療を強化したが，第 2 再発をきたしてその後の化学療法にも抵抗性を示した．家族の同意の下，非寛解期に HLA DNA 型 DRB 1 座不一致のドナーから非血縁者間同種骨髄移植を施行したが拒絶を認め，HLA 血清型 GVHD 方向 2 座，拒絶方向 3 座不一致の母親から再移植を施行した．その結果生着を認めたものの，急性移植片対宿主病 grade 3，thrombotic microangiopathy，veno-occlusive disease を合併し，多臓器不全により再発後 5 カ月で死亡した．症例 2 は順調に化学療法のみで再寛解に至り，以後 8 クールの強化療法を行って寛解を維持していたが，HLA 一致の同胞が存在し，SCT が治療の選択肢となった．しかしながら経時的な骨髄の MRD 量が再寛解後すべて測定感度以下で陰性であったことから（Fig. 3），患者家族と相談した結果，移植は実施せず，CCLSG ALL2004 研究の標準危険群維持療法（Fig. 1）を 9 カ月間行い治療終了とした．再発治療終了後 29 カ月現在 MRD 陰性のまま寛解を維持している．

症例 3 と 4（S3 群）も，同じく ALL-REZ BFM2002 研究に基づいて再寛解導入化学療法を実施し，骨髄にて再寛解が確認された．しかし Point 1 および Point 2 での MRD 量はそれぞれ 10^{-2} と 10^{-3} で残存を認めたため（Fig. 3），SCT の適応となった．いずれも血縁ドナーが得られず非血縁者間移植の準備を進めていたが，症例 3 は再寛解後約 5 カ月で第 2 再発を来した．その後は各種治療に抵抗性となり，再発後約 13 カ月で腫瘍死した．症例 4 は強化療法 4 コース施行後，HLA 完全一致の非血縁ドナーより同種骨髄移植が施行された．移植後 MRD も測定感度以下となり，現在まで寛解を維持している．

また，症例 1，3，4 については過去の余剰検体が解析可能であったため，これらを用いて後方視的に MRD 定量を行った．症例 1 は再発 39 カ月前（初回治療開始後 7 カ月），症例 4 は 7 カ月前（初回治療開始後 14 カ月）の時点ですでに MRD の陽性化が認められ，症例 3 は再発 6 カ月前に陰性ではあるものの 10^{-4} で増幅が認められていた．

Table 3 Ig/TCR gene rearrangement and sequence of ASO primer of each case

	Gene rearrangement		Sequence of ASO primer
Case 1	TCRγ (Vγ1-Jγ1.1)	Vγ1: del 18bp Jγ1.1: del 5bp ins 4bp	TTGATCCAATCACT*CCCGG*TAATAGACC
Case 2	Igκ (κDE-RS)	κDE: del 3bp RS: del 3bp ins 4 bp	TAGCCAGCTTTCCTG*TTCC*GCCCTAGTGG
Case 3	Igκ (κDE-Vκ1)	κDE: del 0bp Vκ1: del 1bp ins 2bp	ACTTACAATGCCCCCCGGAGCCCTAGTG
Case 4	TCRγ (Vγ1-Jγ1.3)	Vγ1: del 5bp Jγ1.3: del 12bp ins 7bp	TGTGCCACCTGGG*CCCGGA*GGAAACTCT

Italic letters indicate the inserted nucleotides. Arrow indicates sequence of ASO primer from 3′ to 5′. del: deletion, ins: insertion.

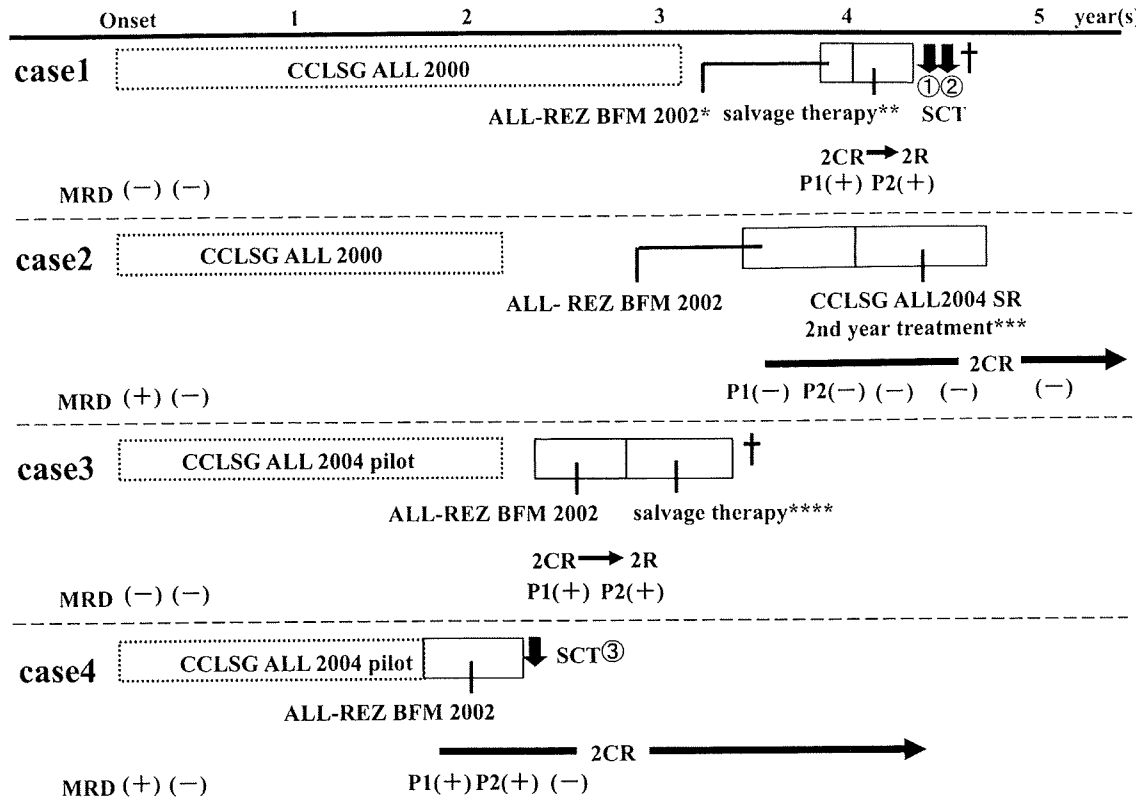

Fig. 2 Clinical course and the results of PCR-based MRD analysis of each case
* ALL-REZ BFM 2002 protocol consists of dexamethasone, 6MP, VCR or vindesine, HD-MTX, HD-AraC, ifosmide, daunorubicin, L-Asp, and triple IT. ** Salvage therapy for case 1 consists of HD-AraC＋CPM＋THP-ADR＋PEG (polyethylene glycol)-Asp＋PSL, HD-AraC＋etoposide＋idarubicin, and AraC＋mithoxantrone＋etoposide. *** CCLSG ALL 2004 SR 2nd year treatment is shown in Fig. 1. **** Salvage therapy for case 3 consists of HD-AraC＋CPM＋THP-ADR＋L-Asp＋vindesine＋dexamethasone, rituximab＋L-Asp, topotecan＋vinorelbine＋thiotepa＋gemcitabine＋dexamethasone, and etoposide＋AraC＋idarubicin. Conditioning regimens of SCT are as follows, SCT①: melphalan＋total body irradiation, SCT②: fludarabine＋antithymocyte globulin＋total body irradiation, SCT③: etoposide＋CPM＋total body irradiation. P1 (Point 1) and P2 (Point 2) are the points of MRD quantification shown in Fig. 3. 2CR: second complete remission, 2R: second relapse.

IV. 考　　察

Ig/TCR 遺伝子再構成を利用した PCR-MRD 定量は1980年代後半より開発が進み，形態診断の100倍以上と考えられる 10^{-4} から 10^{-5} の感度で MRD の検出が可能で，初発 ALL では寛解導入後の結果が独立した予後因子となることが証明され[6-8]，BFM グループや Dana-Farber Cancer Institute[7] でこの定量結果を用いた治療層別化がなされている．これは，リンパ球系細胞が Ig/TCR 遺伝子再構成やランダムな塩基の挿入によって多様性を獲得した後単クローン性に自律増殖して腫瘍化することを利用して，そのクローン特異的な塩基配列を PCR 増幅し分子生物学的マーカーとする手法である[17]．本邦においては，CCLSG ALL 911 研究における後方視的研究で岡本らが MRD 定量値に反映される初期治療反応性と予後との相関を報告しており[9]，CCLSG では今回の症例に使用された ALL 2000 MRD 研究以降，治療層別化の因子に用いられている[10]．

一方，再発 ALL における PCR-MRD 定量についても，やはり BFM グループで ALL-REZ BFM として研究が続けられており，前述の如く再発症例を再発時期，再発部位，白血病細胞の免疫学的表現型で4群に層別化する S 分類（Table 2）[11] を用い，S2 群で後方視的研究が行われている．これは ALL-REZ BFM90, 95, 96 研究における S2 群に関して MRD 解析結果と予後との相関を検討したもので，day 36（再寛解導入療法終了後）の MRD 陰性例16例の6年無イベント生存率（event-free survival: EFS）が86%であるのに対して MRD 陽性例10例は0%であったことから，MRD 定量によって確認できる早期の治療反応性が長期予後の予測因子であると述べられて

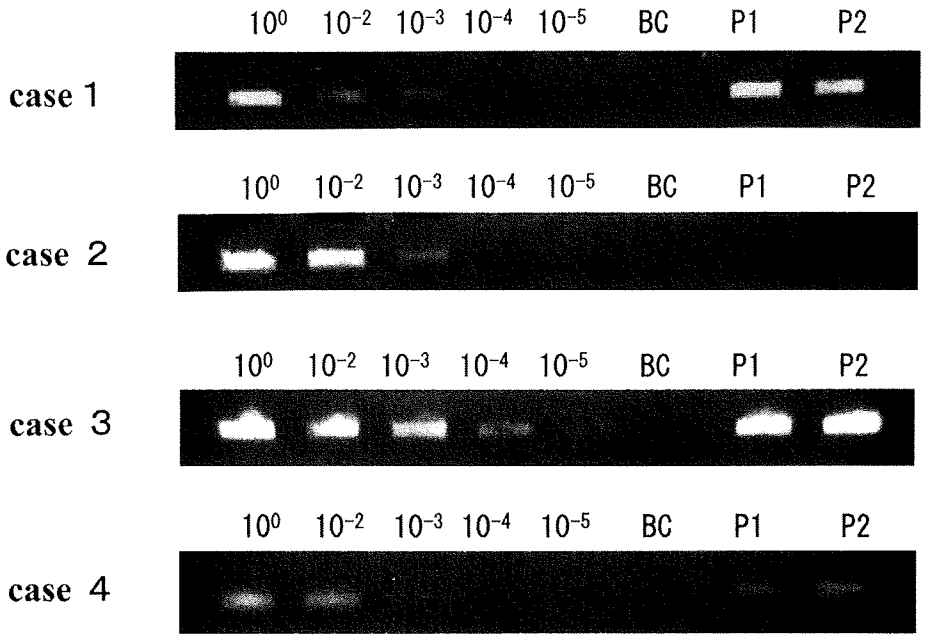

Fig. 3 Results of MRD quantification by nested PCR
The bone marrow DNA sampled at relapse (10^0) with the serially (with 10-fold dilution steps, 10^{-2}–10^{-5}) diluted the leukemic DNA in the BC (buffy coat) and the bone marrow DNA after treatments (P1 and P2) were amplified using allele-specific primers (see Table 3) with BC as a normal DNA control.

いる[8]．これを受けて現行の 2002 研究では，MRD 定量値によって S2 症例を化学療法単独と SCT に層別化を行っており，今回われわれが報告した S2 群の 2 症例は，この治療戦略に基づいて治療方針を決定した．MRD 陽性例は SCT にても救命できなかったが，MRD 陰性例については，現時点で寛解を維持しており，従来であれば HLA 一致同胞の存在から SCT を選択したであろうが，MRD 定量の導入によって移植関連毒性や晩期障害といった SCT に伴う危険を回避できたと考えられる．このように本例は，分子生物学的 MRD 定量に基づく再発治療法の選択が本邦でも十分に実施可能であることを証明する貴重な症例であるといえる．

また S3 群については，ALL-REZ BFM87 研究では 15 年 EFS が 18%[3] との報告があり，83 研究から 96 研究のまとめでは，各群の EFS が S1：75%，S2：38%，S3：2%，S4：4%と明らかな有意差をもって S3/4 群の難治性が示されている[17]．われわれの S3 群の 2 症例でも，再寛解には至ったものの MRD は依然として陽性であり，死亡した 1 例は SCT 前に第 2 再発をきたした．今回の S3 群での MRD 定量結果は，この群の治療抵抗性と強力な治療の妥当性の裏付けになると考えられた．

本邦の再発 ALL の全体の成績は，Yumura らが 157 症例の解析で 5 年 EFS 32.9%[18]，Matsuzaki らが 117 例の解析で EFS 25.1%[19] とそれぞれ報告している．今後治療成績の向上のためには，S3/S4 症例に対する治療の検討をはじめさまざまな課題が考えられるが，とくに S2 群に存在すると考えられる予後良好で SCT を避けられうる集団を抽出することは，患児の負担や晩期障害の軽減の意味でも重要であると考えられ，この目的のためには PCR-MRD 定量は非常に有用な手段である．また，化学療法中や終了後あるいは SCT 後の治療効果判定としても，この手法を用いていわゆる‘寛解の深さ’を確認することが可能であり，当科および CCLSG 参加施設を中心としたさまざまな症例で実用化されている．さらに，再発前の保存検体の後方視的な解析により，症例 3，4 で第一寛解期に MRD の増加がみられていたことから，再発クローンが初発時と同一の場合には，再発の早期発見や再発予防に PCR-MRD を応用できる可能性が考えられた．

MRD 定量にはこのほかに，FCM による免疫学的手法があり，その利点は比較的安価で，定量性と迅速性にすぐれ，多くの施設に機器が普及しているため導入が容易であることなどが挙げられる．しかしながら正常細胞集団との区別が困難な場合，データの特異性に問題が生じることがある．これに対して PCR-MRD 定量は検出感度が FCM の約 10 倍で症例特異性が高く，スメアなどの保存検体でも解析が可能，などの利点をもつ反面，高価で手技的に煩雑である．また症例 1 のように再発時に

クローンが変化した場合には結果が偽陰性となってしまうため，複数の再構成によるフォローアップで診断精度を高める必要がある．両者の相関についても報告があり，その組み合わせによって，より確実なMRDの定量が実施されている[20]．

以上のようにPCR-MRD定量は，欧米ではすでにその有用性と実用性が確立され，リンパ系腫瘍の臨床の場でさまざまな活用が期待されるため，今後国内でもより多くの症例で積極的に施行されるべきと考えられた．

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３．混合型白血病
～診断の現状と問題点～

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Summary　小児急性白血病において免疫学的診断の果たす役割は大きく，初期治療の選択には欠かせない検査である。しかし近年の知見によりその重要性や意義は変化し，診断基準の改定と混合型白血病の定義を改訂する必要が生じてきた。そこで我が国において過去に行われた小児白血病マーカー検査結果を後方視的に解析し，現状に合わせた新しい診断基準を作成した。すでにいくつかの臨床試験でこの診断基準が用いられている。これらの診断基準を用いることで新たなリスク因子の抽出に役立つよう，我が国における免疫診断の基盤整備が望まれる。

はじめに

小児白血病は小児がんのおよそ半数を占めており，年間およそ700例前後の発症があると考えられている。かつては不治の病であったが，近年は治療成績が極めて向上し，70～80%，あるいはそれ以上の長期生存が期待できるようになった。これは臨床試験を実施し，年齢や白血球といった予後因子を見出し，治療の改変に応用して治療成績の向上を得たからである[1]。予後因子には白血病の病型，すなわち免疫学的分類も含まれている。かつては形態学を併用した骨髄性・リンパ性の区別のみであったものが，モノクローナル抗体やフローサイトメトリー（FCM）の発展に伴ってＴ細胞性・Ｂ細胞性，そしてさらにその亜分類と，免疫学的分類は初期治療の選択に欠かせない存在となった。さらに最も強力な予後因子である治療早期反応性の評価もこの免疫学的診断を応用して，主としてFCMで測定されている[2]。また正確な免疫学的診断は，FCMを用いた微小残存病変（MRD）の検出にも役立っている[3]。

小児白血病治療はすでに高い長期生存率を示している臨床試験であり，予後良好群においては治療軽減が，予後不良群では治療強化が必要とされるため，各群の正確な抽出作業が必須である。また予後良好であるが故に，統計的有意差を得るためには大規模な臨床試験が必要となる。しかし研究規模が大きくなればなるほど，診断や治療での施設間格差の危険性が生じる。精度の高い標準的な診断法が確立され正確な診断を得て初めて，最

FCM（フローサイトメトリー）　MRD（微小残存病変）

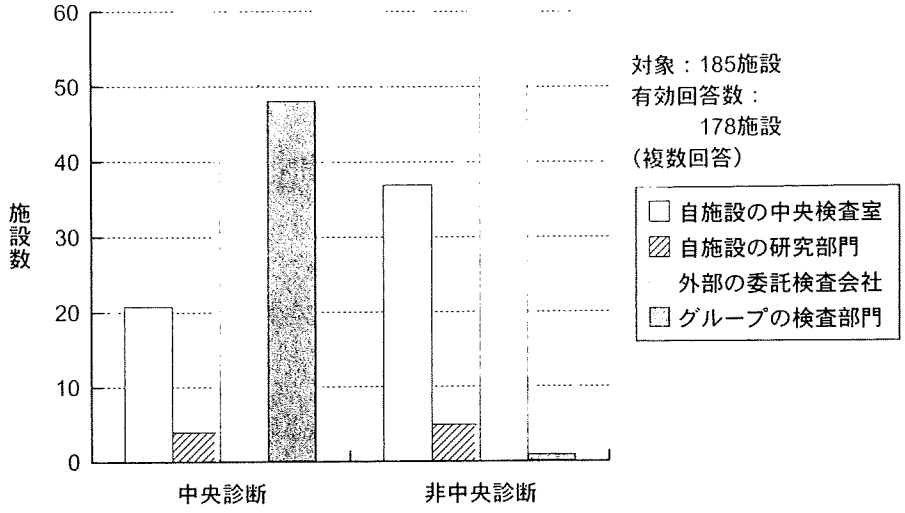

図1　小児白血病診断におけるマーカー検査依頼先

小児白血病マーカー診断の依頼先について行ったアンケート調査結果。中央診断を行っている研究グループに属する施設と行っていないグループの施設について，各々の依頼先を示した（複数回答可）。（筆者作成）

も効果的な治療法の選択が可能となると言える。

平成14年度から厚生労働科学研究費補助金「効果的医療技術の確立推進臨床研究事業における小児造血器腫瘍の標準的治療法の確立に関する研究」が開始され，初発時における治療法選択に必要な小児造血器腫瘍の免疫学的診断の標準化を目的に「免疫学的診断の標準化ワーキンググループ(**免疫 WG**)」が結成された。平成19年度までに免疫学的診断の実態調査，マーカー解析パネル案の作成，外部精度管理，我が国における「急性リンパ性白血病の免疫学的診断基準（案）」の作成を行った。現在は全国規模の小児造血器腫瘍共同治療研究の開発を目的に設立された小児白血病リンパ腫研究グループ（JPLSG）の免疫診断委員会として，各種臨床試験における診断基準や解析パネルの策定，最終診断の決定に参画している。

これらの活動を通して得られた小児白血病の免疫診断，とりわけ混合型白血病の診断に対する知見と展望につき解説したい。

1．小児白血病の免疫診断の現状と問題点

平成16年度に免疫WGによって実施された小児白血病の免疫診断の現状に対するアンケート調査では，小児白血病診断における細胞表面マーカー検査の依頼先は，中央診断を行っている治療研究グループの治療施設では研究グループにおける解析センターへの依頼が最も多く，臨床検査会社，自施設の順に依頼されていた。中央診断施設をもたない研究グループの場合には臨床検査会社への依頼が多く，ついで自施設へ依頼されていた[4]。ただ，いずれの場合も検査は1カ所のみではなく，複数の検査機関へ依頼されていることが

免疫WG（免疫学的診断の標準化ワーキンググループ）　JPLSG（小児白血病リンパ腫研究グループ）

表1　急性白血病の免疫学的診断に有用なマーカー解析パネル（案）

Primary panel

B細胞系	T細胞系	骨髄系	non-lineage
CD19	細胞表面 CD3	MPO	CD10
CD79a	細胞質内 CD3	CD13	CD34
κ / λ	CD7	CD33	CD45
		CD41	CD56
			HLA-DR
(16 種類)			TdT

Secondary panel

B細胞系	T細胞系	骨髄系	
CD20	CD2	CD14	CD15
CD22	CD4	CD65	CD36
細胞表面 IgM	CD5	CD117	CD42b
細胞質内 μ 鎖	CD8	GlycophorinA	CD61
			CD64
(17 種類)			

追加パネル

B細胞系	T細胞系
CD24	CD1a
CD58	TCR α / β
	TCR γ / δ
(5 種類)	

(JPLSG 免疫診断委員会作成)

多かった（**図1**）。この現状を踏まえて，平成17年度以降にJPLSGを母体として開始された小児白血病治療研究では，全症例で免疫WG推奨パネル（**表1**）に準拠したマーカー中央診断が開始されることとなった。現在，成育医療センター（主に東京小児がん研究グループ：TCCSG，および九州山口小児がん研究グループ：KYCCSG），愛知医科大学（小児がん白血病研究グループ：CCLSG），三重大学（主に日本小児白血病研究会：JACLSおよびCCLSG），大阪大学（JACLS）の4施設において，統一した診断パネル・抗体クローンと，「フローサイトメトリーによる造血器腫瘍細胞表面抗原検査に関するガイドライン」[5]に基づいたサンプル調整法を用いて中央診断を実施している。**表2**に現在までにマーカー中央診断が実施されている，あるいは中央診断を計画中の臨床試験を示す。

アンケート結果から患者骨髄検体はこれら中央診断と，自施設もしくは検査会社の2カ所へ検体を提出していると考えられる。その理由として，

表2　JPLSG診断基準でマーカー中央診断実施中および実施予定の小児白血病臨床試験

実施中
- 急性骨髄性白血病：AML-05
- 再発急性リンパ性白血病：ALL-R08

実施予定
- 乳児急性リンパ性白血病：MLL-10
- T細胞性白血病：ALL-T10
- B前駆細胞性白血病：ALL-B10

(筆者作成)

自施設や検査会社での検査には保険適応があること，中央診断より迅速に結果が得られること，複数の検査施設に提出することで検査結果の信頼性を高めたいことなどが考えられる。その一方，とりわけ検査会社におけるマーカー検査は保険適応範囲内で行うため，ほとんどの場合，抗原検索数は最大でも18項目以内となっており，また細胞質内抗原の検索は通常実施されない。そのため，とりわけ混合型白血病の診断には十分な項目数が

表3　JACLSにおけるALL診断基準

1) B-lineage：
　B-precursor：CD19 & HLA-DR陽性かつIgG、IgM、Igκ、Igλ陰性
　ただし、cytoplasmic IgM陽性はPre-B ALL
　Mature B (B-ALL)：細胞表面Ig陽性
2) T-lineage：CD2、3、5、7、8の2つ以上陽性
3) Acute mixed-lineage leukemia (AMLL)：
　Type 1：T-ALLの基準を満たし、CD13、14、33の2つ以上が陽性
　Type 2：T-ALLの基準を満たし、CD13、14、33の1つ以上とCD19、20、22の1つ以上が陽性
　Type 3：B-pre ALLの基準を満たし、CD2、3、5、7、8の1つ以上とCD13、14、33の1つ以上が陽性
4) Acute unclassified leukemia (AUL)：上記のどの分類にも当てはまらないもの

(JACLS ALL委員会作成)

検討されていない場合がほとんどである。また，結果の報告は通常解析データのみであり，免疫学的診断が付記されて報告されることは稀である。

現在実施されている中央診断は，混合型白血病に対応できる十分な項目数の検索がされること，免疫学的診断を記載して報告されることが利点である。加えて患者同意の元に余剰検体を凍結細胞として一括して保存できることもメリットである。一方，保険診療外の検査であり，検査費用を診療報酬へ求めることは現状では不可能である。また，検査会社のような検体搬送システムを有さず専ら宅配便での検体搬送となることから，搬送中の検体劣化の危険性は否定できない。マーカー診断のみならず保険適用外であるほとんどの遺伝子検査も含めて，検体保存を含めた包括的診断・検体保存システムの構築は，小児白血病領域において質の高い臨床試験を実施していく上で極めて切迫した課題であると言える。

2．混合型白血病診断の現状と問題点

我が国における混合型白血病の診断は，これまで個々の治療研究グループによって決定されており，独自の基準を用いたり，EGIL基準を用いたりされてきた。**表3**にこれまでJACLSにおいて使用されてきた急性リンパ性白血病(**ALL**)における混合型白血病の基準を示す。しかしながらこれらの診断基準では，例えばCD2を発現するB前駆細胞性白血病(**BCP-ALL**)をCD13やCD33の発現によってしばしば混合型白血病と診断し治療強化が行われていたが，CD2を発現するALLの予後は比較的良好であるとの報告もあり，臨床的予後を反映しているかは疑問が残るものであった[6]。

近年，プレドニン単剤治療への反応性[7]や，治療開始1週間での骨髄中芽球割合を用いた治療早期反応性によるリスク分類[2]が広く利用されるようになったこと，また分子遺伝学的検査の発達により，混合型白血病診断の重要性が変化してきた。すなわち抗原発現によって混合型白血病を分類しようとするよりも，初期治療選択のためにどの分化系統に属しているかを決定することが重要となり，最終的な治療法選択は治療への反応性や，分子遺伝学的検査を用いて変更することが一

ALL (急性リンパ性白血病)　BCP-ALL (B前駆細胞性白血病)

般的となった。新たにWHOによる分類が提唱されることとなった理由のひとつと考えられる。

WHO分類における混合型白血病の診断[8]は，「acute leukemia of ambiguous lineage」として記載されており，**表4**に示す各lineageにおける定義を単一クローンで複数満たすことが必要である。この診断基準は普遍的で広く用いることができるよう意図されているが，我が国で実際に用いる上では多少の問題がある。すなわち診断基準の項目中にNSEやlysozymeといった項目が含まれているが，現状ではフローサイトメトリーで簡便に利用できる抗体が存在せず，生検組織や骨髄クロット標本を常に作成し，混合型が疑われる場合には免疫組織学的検討が必須となることが問題となる。WHO分類においてMyeloid lineage（MPO陽性）のみでなく単球系抗原による分類を加えることは，我が国では検討された実際のデータが存在しないことや，欧米からも明確な報告を実際には見いだせないことから，現時点では必要性が疑問視される。また近年，白血病の発生起源となる前駆細胞についての知見が明らかになるにつれ，T細胞性と単球系，あるいはB細胞系と単球系の共通幹細胞が各々存在するが，T細胞とB細胞の共通幹細胞は存在しないという説が有力となりつつある[9,10]。実際，MLL遺伝子再構成を有する乳児白血病の一部はB細胞系と単球系のbiclonal leukemiaを示すことがあり，B細胞系と単球系の共通幹細胞からの発症と考えられている。このいわゆる「ミエロイド基本型モデル」が正しいとすれば，T細胞もしくはB細胞とMyeloid lineage（すなわちMPO陽性），あるいはT細胞とB細胞の診断基準を同時に満たす場合のみが真の混合型白血病であると考えることができる。

その他，WHO分類中に示されるMLL遺伝子やt（9；22）に関連する病態は，細胞遺伝学的検査の結果が発症時には得られていないため，最終診断として用いることは可能であっても，初発時からそれらと診断することは容易ではない。

表4　WHO分類における血球分化系統診断

骨髄系 MPO陽性（FCMもしくは細胞化学）もしくは単球系分化（NSE、CD11c、CD14、CD64、lysozymeのうち少なくとも2つ）
T細胞系 細胞質内CD3（CD3ε鎖への抗体によるFCMでの確認）もしくは表面CD3
B細胞系 CD19強陽性かつCD79a、細胞質内CD22、CD10のうち少なくとも1つが強陽性 CD19弱陽性かつCD79a、細胞質内CD22、CD10のうち少なくとも2つが強陽性

（WHO分類に基づき筆者作成）

以上のことから，我が国の小児白血病臨床試験でWHO分類をそのまま利用することは困難であり，我が国の現状に即した診断基準の作成が必要と考えられた。

3．新しい混合型白血病の診断基準

平成19年度に免疫WG（現：JPLSG免疫診断委員会）は，Campanaらによる報告[11]を参考に我が国の小児白血病における実際のマーカー検査結果を後方視的に検討した。診断基準作成に十分な項目が解析された合計2,321例（T-ALL 224例，BCP-ALL 1,451例，pre-B ALL 165例，B-ALL 44例，AML 372例，Mixed lineage/unclassified leukemia：AUL 65例）の検討から，中央診断を実施する検査施設の実情や最新の知見を元に，新しく小児ALL（**表5**）と混合型白血病（**表6**）の診断基準案を作成した。

これらの基準は，①初発時の免疫診断での利用，②我が国で実際に利用されている抗原に基づいた分類，③可能な限り帰結先を決定する，④国

際的にも通用する，を念頭に作成されている。急性骨髄性白血病(AML)においては，マーカー診断の重要性がやや低いこと，マーカー診断と形態あるいは遺伝子診断との関連が必ずしも明確でないことから，発現パターンを示すに留めた。また，NK/Myeloid leukemia/lymphomaについては，診断基準を作成するために十分な検討が今までなされていない(とりわけM7との鑑別)ため，診断基準は提示されていない。また，2系統以上のクローンを有する白血病においては，各々のクローンについて診断をすることとした。

新しい診断基準を用いてMixed lineage/AULと診断された65例の再分類を試みたところ，T-ALLの診断基準を満たすAMLと考えられる3例と，Biclonalと考えられた1例を除き再分類は可能で，分類不能例はなかった。また，T-ALLとB細胞系ALLの診断基準を同時に満たす症例(True Mixed ALL)は存在しなかった。以上より，小児白血病診断におけるこのJPLSG診断基準の有用性が示唆された。今後，小児白血病の大部分を占める初発ALLの臨床試験においてこの診断基準の有用性が検証されるとともに，診断基準の見直しが行われる予定である。

表5　JPLSGにおけるALLの診断基準

CD3陽性かつ，CD2，CD5，CD7，CD8のうち1つ以上陽性：T-ALL
CD19，CD79a，CD20，CD22のうち2つ以上が陽性かつ， ・細胞質内μ鎖，Igκ，Igλがすべて陰性のものをB-precursor ALL ・細胞質内μ鎖陽性でIgκ，Igλがともに陰性のものをpre-B ALL ・Igκ またはIgλ陽性のものを成熟B細胞性ALL

(JPLSG免疫診断委員会作成)

おわりに

混合型白血病の検討は，白血病細胞の起源を考える上で非常に重要である。AMLにおいては白血病幹細胞の存在が示されつつあるが，急性リンパ性白血病では未だ明らかではない。正確な免疫診断を実施することが，白血病細胞の起源・分化・増殖機構の解明に大きな手がかりを与えることが期待される。また近年のFCM技術の発展が，混合型白血病診断とともに新規抗原をはじめとする種々のリスク因子同定，MRDのフォローに役立つことも期待される。

しかしながら先に述べたように，我が国におい

表6　JPLSGにおけるMixed lineage leukemiaの分類

骨髄抗原陽性B細胞系ALL	リンパ系抗原陽性AML
1．CD19，20，22，もしくは79aの1つ以上が陽性，かつ 2．$CD3^-$ かつ 3．MPO^-で，CD13，15，33，または65が陽性	1．MPO^+，もしくはCD13，15，33，65の1つ以上が陽性，かつ 2．$CD3^-$ かつ，$CD79a^-$ かつ 3．CD2，5，7，19，22，もしくは56が陽性
骨髄抗原陽性T-ALL	**True mixed lineage leukemia**
1．T-ALLの診断基準を満たし，かつ 2．$CD79a^-$ かつ 3．MPOで，CD13，15，33，または65が陽性	1．MPO^+ かつB細胞系の診断基準を満たす，もしくは 2．MPO^+ かつ，T-ALLの診断基準を満たす，もしくは 3．B細胞系とT-ALLの両方の診断基準を満たす

(JPLSG免疫診断委員会作成)

AML(急性骨髄性白血病)

て白血病免疫診断におけるインフラは極めて貧弱である。混合型白血病という稀な病態の診断はおろか，臨床の現場で必須となる免疫学的診断に関する基本的な情報でさえ，現在の保険診療範囲内では実施不可能である。免疫学的診断のみならず，DNA や RNA レベルでの遺伝子診断，余剰検体の保存と使用，臨床経過データ等，包括的に管理・利用できるシステムの構築は，我が国の治療成績を世界に発信し，基礎研究において新しい知見を得ていくためには不可欠である。今後の発展を切に期待したい。

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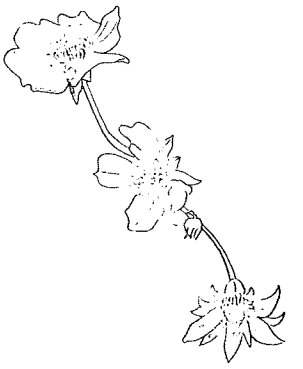

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Positive auto-regulation of *MYCN* in human neuroblastoma

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ABSTRACT

MYCN oncogene is one of the most important regulators affecting the prognosis of neuroblastoma and is frequently amplified in the high-risk subsets. Despite its clinical significance, it remains unclear how the *MYCN* expression is regulated in human neuroblastomas. Here, we found the presence of a positive auto-regulatory mechanism of *MYCN*. Enforced expression of MYCN induced endogenous *MYCN* mRNA expression in SK-N-AS neuroblastoma cells with a single copy of *MYCN* gene. Luciferase reporter assay revealed that MYCN protein activates its own promoter activity in a dose-dependent manner and the downstream region relative to the transcription start sites is responsible for the activation. Furthermore, ChIP analysis showed that MYCN is directly recruited onto the intron 1 region of *MYCN* gene which contains two putative E-box sites. Intriguingly, in response to all-*trans*-retinoic acid (ATRA), MYCN was down-regulated in *MYCN*-amplified SK-N-BE neuroblastoma cells, and the recruitment of MYCN protein onto its own intron 1 region was reduced in association with an induction of neuronal differentiation. Collectively, our present results suggest that MYCN contributes to its own expression by forming a positive auto-regulatory loop in neuroblastoma cells.

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Introduction

Neuroblastoma is one of the most common solid tumors in children and originates from the embryonic neural crest cells [1,2]. It accounts for about 15% of childhood cancer deaths, and at least 40% of all neuroblastomas are designated as high-risk tumors which often occur in patients over one year of age and show characteristic genomic abnormalities including allelic loss of the distal part of chromosome 1 and gain of chromosome 2p [1–3]. *MYCN* is an oncogene mapped to chromosome 2p, and its amplification is a strong indicator for poor outcome in patients' survival [4–6]. Transgenic mice which overexpress *MYCN* driven by the tyrosine hydroxylase promoter in sympathetic neurons develop aggressive neuroblastoma, indicating that *MYCN* has an intrinsic oncogenic potential *in vivo* [7]. MYCN protein activates transcription of the genes that are involved in diverse cellular function, such as cell growth, apoptosis, and differentiation [8,9]. Recently, by using computational analysis of gene expression profile, Fredlund et al. showed that high transcriptional activity of MYC family pathway in primary neuroblastomas predicts poor outcome of the patients and is correlated with low grade of neuronal differentiation in tumors [10]. These results are consistent with the notion that MYCN promotes tumor progression via transcriptional activation of the target genes, and that down-regulation of MYCN may be a critical step for the process of neuronal differentiation. Indeed, in response to differentiation stimuli like NGF or retinoic acids (RAs), endogenous expression of MYCN is suppressed [11,12] and enforced expression of MYCN inhibits NGF- or retinoic acid-mediated neuronal differentiation [13,14]. Moreover, MYCN silencing alone is enough to induce neuronal differentiation in several *MYCN*-amplified neuroblastoma cell lines [15,16].

Retinoic acids (RAs) are now being used as one of the tools in the standard treatment protocols for high risk neuroblastomas, and demonstrated significant therapeutic effects on event-free survival [1,17,18]. Although the down-regulation of MYCN by RA was first reported in 1985 [12], the precise mechanism of its regulation is still elusive [19]. Previously, E2F family and Sp1/Sp3 were reported as the transcription factors which regulate basal expression of MYCN [20–22]. In response to retinoic acid treatment, binding of E2F-2, -3 and -4 to the core promoter of *MYCN* gene was decreased after 12 days of RA treatment [20], whereas Sp1/Sp3 binding was not affected by RA [23]. However, since RA treatment represses *MYCN* transcription at more early stage [12], there might

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be alternative mechanisms which accelerate a decline of *MYCN* mRNA in response to RA treatment.

In the present study, we have found the presence of a positive auto-regulation in *MYCN* transcription. MYCN protein enhances its own promoter activity through its direct recruitment onto the intron 1 region of *MYCN* gene. Treatment with all-*trans*-retinoic acid (ATRA) significantly represses *MYCN* mRNA expression accompanied by a marked decrease of the amount of MYCN protein recruited onto the intron 1 region. These results suggest that the positive auto-regulation of MYCN is repressed by ATRA, resulting in the further down-regulation of *MYCN* mRNA expression.

Materials and methods

Cell culture and transfection. SK-N-AS neuroblastoma cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and antibiotic mixture in a humidified atmosphere of 5% CO_2 in air at 37 °C. SK-N-BE neuroblastoma cells were cultured in a mixture of minimal essential medium (MEM) and Hanks F12 medium supplemented with 15% heat-inactivated FBS and antibiotics. For transfection, cells were transfected with the indicated expression plasmids using LipofectAMINE 2000 according to the manufacturer's instructions (Invitrogen).

Construction of luciferase reporter plasmids. A luciferase reporter plasmid containing the region of *MYCN* promoter encompassing from −221 to +1312 (where +1 represents the transcription initiation site) was generated by PCR-based amplification using genomic DNA prepared from human placenta as a template. Oligonucleotide primers used were as follows: 5′-GAGCTCCAGCTTTGCAGCCTTCTC-3′ (forward) and 5′-AACCAGGTTCCCCAATCTTC-3′ (reverse). An underlined sequence in the forward primer indicate the SacI restriction site. PCR products were subcloned into pGEMT Easy plasmid (Promega) according to the manufacturer's protocol. After sequencing, PCR products were digested with SacI and subcloned into SacI restriction sites of pGL3 basic plasmid (Promega) to give MYCN(−221/+1312).

RT-PCR and quantitative real-time RT-PCR. Total RNA was prepared using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. cDNA was synthesized using SuperScript II with random primers (Invitrogen). Quantitative real-time RT-PCR using an ABI PRISM 7500 System (Perkin-Elmer Applied Biosystems) was carried out according to the manufacturer's protocol. Following were primers used for this analysis: human *MYCN* 5′-TCCATGACAGCGCT AAACGTT-3′ (forward) and 5′-GGAACACACAAGGTGACTTCAACA-3′ (reverse). All the reactions were performed in triplicate. The mRNA levels of each of the genes were standardized by β-actin.

Immunoblotting. Cells were washed with ice-cold PBS and lysed with SDS-sample buffer. After a brief sonication, cell lysates were boiled for 5 min, resolved by 15% SDS–PAGE, and electrotransferred onto Immobilon-P membranes (Millipore). The membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% nonfat dry milk, and then incubated with monoclonal anti-MYCN (AB1, Oncogene Research Products), monoclonal anti-tTG (AB-3, NeoMarker), anti-TUBBIII (TuJ1, Covance), or with polyclonal anti-actin (20–33, Sigma) antibody for 1 h at room temperature, followed by an incubation with an appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The chemiluminescence reaction was performed using the ECL reagent (Amersham Biosciences).

Luciferase reporter assay. SK-N-AS cells were co-transfected with the indicated *MYCN* promoter luciferase reporters, pRL-TK *Renilla* luciferase cDNA together with or without the increasing amounts of the expression plasmid for MYCN. Total DNA per transfection was kept constant (510 ng) with pcDNA3 (Invitrogen). Forty-eight hours after transfection, firefly and *Renilla* luciferase activities were measured with Dual-luciferase reporter assay system according to the manufacturer's instructions (Promega).

Cell counting. Cells were seeded at a density of 10,000 cells/well in 12-well tissue culture plates. After allowing the attachment of cells overnight, culture medium was replaced with the fresh medium containing with or without 5 μM of ATRA. At the indicated time periods after ATRA treatment, the numbers of viable cells were measured in triplicate under microscopic observation.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed according to the protocol provided by Upstate Biotechnology. In brief, SK-N-BE cells were treated with or without 5 μM of ATRA for 3 days, and cells were cross-linked with 1% formaldehyde in medium at 37 °C for 8 min. Cells were then washed in ice-cold PBS and resuspended in 200 μl of SDS lysis buffer containing protease inhibitor mixture. The suspension was sonicated to an average length of 200–600 nucleotides, and pre-cleared with protein G-agarose beads for 30 min at 4 °C. The beads were removed by centrifugation and the chromatin solution was immunoprecipitated with normal mouse serum (NMS) or with monoclonal anti-MYCN (AB1, Oncogene Research Products) antibody at 4 °C overnight, followed by incubation with protein G-agarose beads for an additional 1 h at 4 °C. The immune complexes were eluted with 100 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$) and formaldehyde cross-links were reversed by heating at 65 °C for 6 h. Proteinase K was added to the reaction mixtures and incubated at 45 °C for 1 h. DNA of the immunoprecipitates and control input DNA were purified and then analyzed by standard PCR. Primers used were as follows: *MYCN*: forward 5′-CTGTCGTAGACAGCTTGTAC-3′, reverse 5′-AACCAGGTTCCCCAATCTTC-3′; *NLRR1*: forward 5′-AAGTT GGATTTGATGACTGATACG-3′, reverse 5′-AGGCAAGAGACCATGTGC AGGAG-3′. *NLRR1* was used as a positive control [24].

Results

MYCN enhances its own promoter activity

To examine whether MYCN could directly regulate its own expression in neuroblastoma cells, human neuroblastoma-derived SK-N-AS cells bearing a single copy of *MYCN*, were transiently transfected with MYCN expression plasmid and the expression levels of endogenous *MYCN* mRNA were measured by semi-quantitative RT-PCR. The primer set used in this study was designed to detect the 3′UTR region of *MYCN* mRNA. Therefore, only the endogenous *MYCN* mRNA was detectable. As shown in Fig. 1A, enforced expression of MYCN significantly induced the endogenous *MYCN* mRNA. Lower panel of Fig. 1A showed the results obtained from immunoblotting experiments. Under our experimental conditions, our antibody against MYCN detected both the endogenous and exogenous MYCN. Similar results were also obtained from the quantitative real-time RT-PCR (Fig. 1B), suggesting that MYCN has an ability to transactivate its own promoter.

To identify the MYCN-responsive region within human *MYCN* genomic sequence, we generated a luciferase reporter plasmid containing *MYCN* genomic fragment encompassing from −221 to +1312, where +1 represents the transcriptional initiation site, termed MYCN(−221/+1312). SK-N-AS cells were transiently co-transfected with the constant amount of MYCN(−221/+1312), *Renilla* luciferase reporter plasmid together with or without the increasing amounts of the expression plasmid for MYCN. As shown in Fig. 1C, enforced expression of MYCN resulted in a significant enhancement of the luciferase activity driven by *MYCN* promoter in a dose-dependent manner. These results strongly suggest that the genomic fragment of *MYCN* (at positions −221 to +1312) contains a MYCN-responsive region(s).

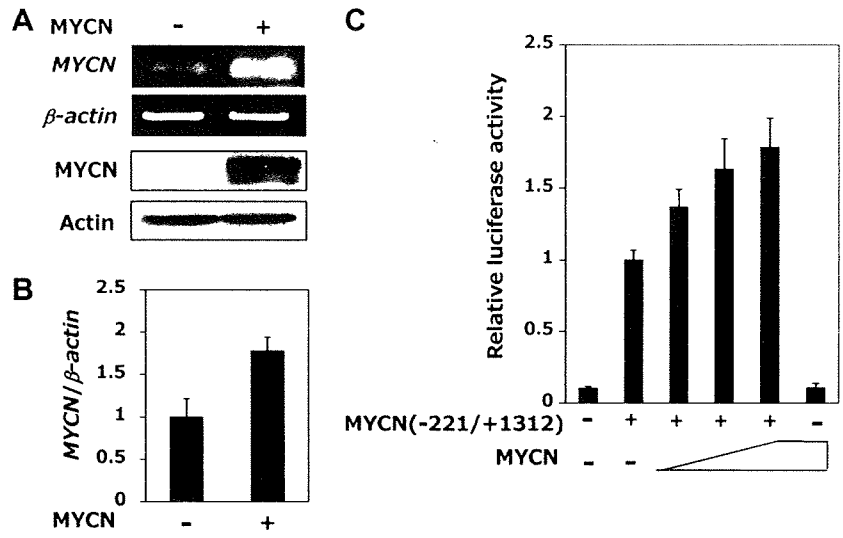

Fig. 1. Enforced expression of MYCN induces the endogenous *MYCN* mRNA. (A) Expression levels of the endogenous *MYCN* mRNA. SK-N-AS neuroblastoma cells were transiently transfected with pcDNA3 or with MYCN expression plasmid. Forty-eight hours after transfection, total RNA and whole cell lysates were prepared and subjected to semi-quantitative RT-PCR and immunoblotting, respectively. For RT-PCR, β-actin was used as an internal control. For immunoblotting, actin was used as a loading control. (B) Quantitative real-time RT-PCR. Total RNA were prepared as in (A) and subjected to quantitative real-time RT-PCR to examine the expression levels of the endogenous *MYCN* mRNA. (C) Luciferase reporter assay. SK-N-AS cells were transiently co-transfected with the constant amount of the luciferase reporter plasmid termed MYCN(−221/+1312) (100 ng) and *Renilla* luciferase reporter plasmid (pRL-TK) (10 ng) along with or without the increasing amounts of MYCN expression plasmid (100, 200, or 400 ng). Forty-eight hours after transfection, cells were lysed and their luciferase activities were measured. Firefly luminescence signal was standardized by the *Renilla* luminescence signal. Results are shown as fold induction of the firefly luciferase activity compared with control cells transfected with the empty plasmid.

MYCN is recruited onto the putative E-boxes located within intron 1 of MYCN to enhance its own promoter activity

During an extensive search for the 5′-upstream region and intron 1 of human *MYCN*, we have found out two canonical E-boxes within intron 1. We then generated two kinds of 3′-trucated *MYCN* promoter luciferase reporter constructs termed MYCN(−1030/+21) and MYCN(−221/+21) and determined their luciferase activities in response to ectopic MYCN. As shown in **Fig. 2A**, the luciferase reporter assay demonstrated that both of those luciferase reporter constructs do not respond to the increasing amounts of MYCN, suggesting that the genomic fragment (at positions +22 to +1312) containing the putative E-boxes but not the 5′-upstream region of *MYCN* is required for MYCN-dependent transcriptional activation of *MYCN*.

To further confirm this notion, we performed a chromatin immunoprecipitation (ChIP) assay. The cross-linked genomic DNA prepared from *MYCN*-amplified human neuroblastoma-derived SK-N-BE cells was subjected to ChIP assay using the indicated primer set (**Fig. 2B**). *NLRR1* which is one of MYCN-target gene [24], was employed as a positive control for this experiment. As clearly shown in **Fig. 2B**, DNA fragment containing the putative E-boxes was specifically amplified, indicating that the endogenous MYCN directly binds to the canonical E-boxes.

ATRA induces neuronal differentiation in SK-N-BE cells

All-*trans*-retinoic acid (ATRA) is one of the well-established inducers for neuronal differentiation and/or apoptosis in neuroblastoma cells. In response to ATRA, a marked reduction in the expression level of *MYCN* is detectable in neuroblastoma-derived cell lines [8]. Consistent with those observations, ATRA treatment resulted in a significant decrease in growth rate of SK-N-BE cells (**Fig. 3A**) in association with their remarkable morphological changes (**Fig. 3B**). Close inspection of cell shapes demonstrated that

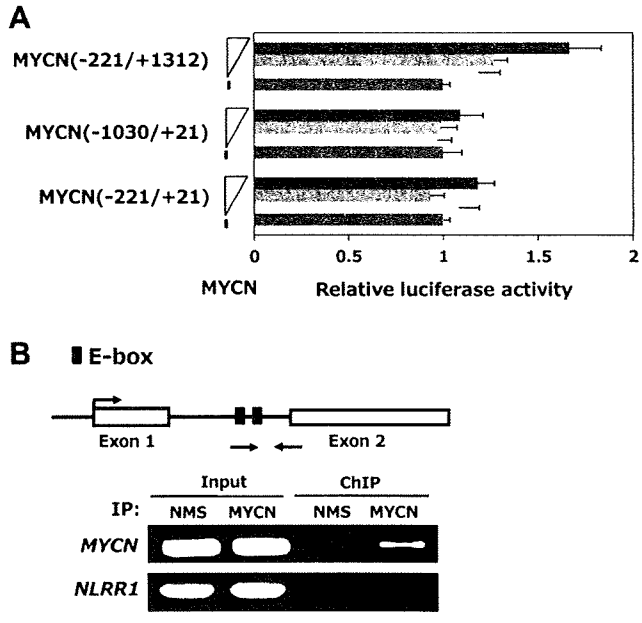

Fig. 2. MYCN has an ability to enhance its own promoter activity. (A) Luciferase reporter assay. SK-N-AS cells were transiently co-transfected with the constant amount of the indicated luciferase reporter constructs (100 ng) and *Renilla* luciferase reporter plasmid (pRL-TK) (10 ng) together with the empty plasmid (pcDNA3) or with the expression plasmid for MYCN. Forty-eight hours after transfection, cells were lysed and their luciferase activities were measured as in **Fig. 1C**. (B) ChIP assay. SK-N-BE neuroblastoma cells were cross-linked with formaldehyde and the cross-linked chromatin was sonicated followed by immunoprecipitation with normal mouse serum (NMS) or with monoclonal anti-MYCN antibody. Genomic DNA was purified from the immunoprecipitates and subjected to PCR using the indicated primer set. The positions of the putative E-boxes and primer set are also shown. The anti-NLRR1 immunoprecipitates were used as a positive control.

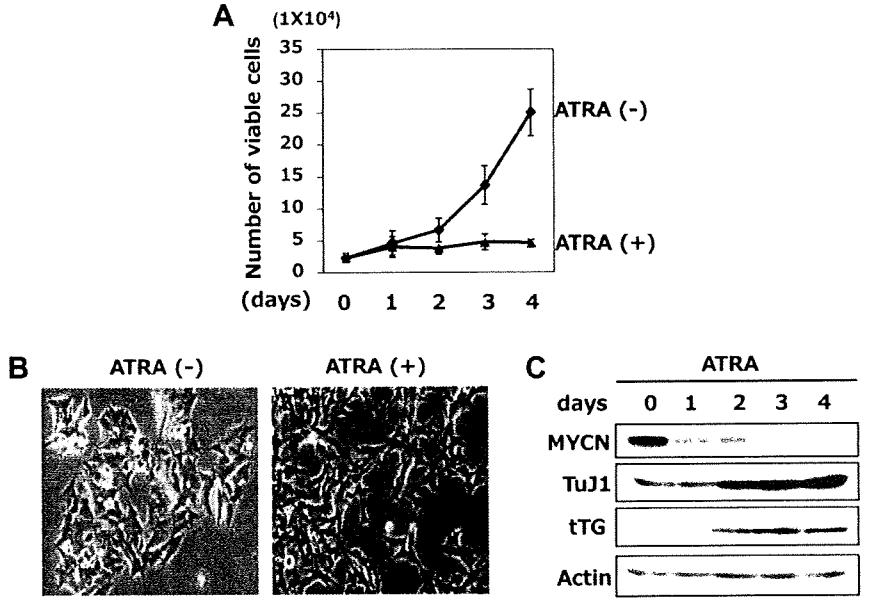

Fig. 3. ATRA induces neuronal differentiation in SK-N-BE cells. (A) Growth curves of SK-N-BE cells in the presence (solid diamond) or absence (solid triangle) of ATRA. Cells were grown in the standard culture medium and treated with 5 μM of ATRA or left untreated. At the indicated time points after the treatment with ATRA, number of viable cells was measured in triplicate. (B) ATRA-mediated neuronal differentiation in SK-N-BE cells. Cells were exposed to ATRA at a final concentration of 5 μM or left untreated. Four days after the treatment with ATRA, cells were examined by phase-contrast microscopy. (C) Immunoblotting. SK-N-BE cells were exposed to 5 μM of ATRA. At the indicated time periods after ATRA treatment, whole cell lysates were prepared and processed for immunoblotting with the indicated antibodies. Actin was used as a loading control.

ATRA treatment induces neurite outgrowth, suggesting that SK-N-BE cells undergo neuronal differentiation in response to ATRA. Additionally, ATRA-mediated down-regulation of the endogenous MYCN and concomitant up-regulation of neuronal markers such as neuron specific class III β-tubulin (TuJ1) [25] as well as transglutaminase II (tTG) [26] were detected as examined by immunoblotting (Fig. 3C).

A significant decrease in the amounts of MYCN recruited onto the genomic region containing the putative E-boxes in response to ATRA

We then examined the expression levels of *MYCN* mRNA in ATRA-treated SK-N-BE cells. To this end, SK-N-BE cells were exposed to 5 μM of ATRA. At the indicated time points after ATRA treatment, total RNA was prepared and processed for quantitative real-time RT-PCR. In accordance with previous observations [12], the expression levels of the endogenous *MYCN* mRNA significantly decreased in ATRA-treated SK-N-BE cells (Fig. 4A, upper panel). Similar results were also obtained from semi-quantitative RT-PCR (Fig. 4A, lower panel). These observations prompted us to examine whether MYCN could be involved in a decrease in *MYCN* mRNA levels in response to ATRA. For this purpose, we performed ChIP assays using ATRA-treated SK-N-BE cells. As shown in Fig. 4B, ATRA treatment remarkably reduced the amounts of the endogenous MYCN recruited onto the genomic region containing the putative E-boxes. Since MYCN enhanced its own promoter activity, our present findings indicate that ATRA-mediated decrease in the expression level of the endogenous MYCN leads to the repression of the positive auto-regulation of MYCN, and thereby promoting neuronal differentiation.

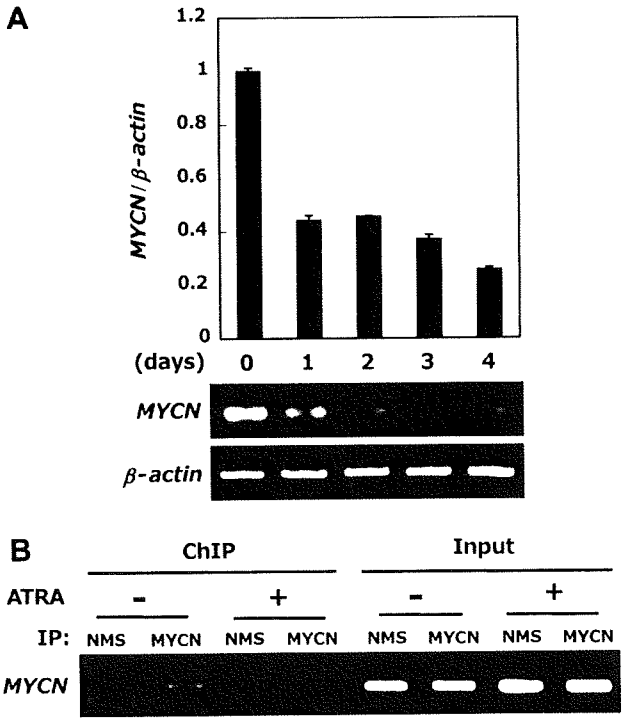

Fig. 4. ATRA-mediated down-regulation of *MYCN* mRNA. (A) Quantitative real-time and semi-quantitative RT-PCR. SK-N-BE cells were treated with 5 μM ATRA. At the indicated time periods after the treatment with ATRA, total RNA were prepared and subjected to quantitative real-time RT-PCR (upper panel) and semi-quantitative RT-PCR (lower panel). *β-actin* was used as an internal control. (B) ChIP assay. SK-N-BE cells were treated with 5 μM of ATRA or left untreated. Three days after ATRA treatment, cells were cross-linked with formaldehyde. The cross-linked chromatin was sonicated and immunoprecipitated with normal mouse serum (NMS) or with monoclonal anti-MYCN antibody. Genomic DNA was then purified from the immunoprecipitates and subjected to PCR-based amplification by using the primer set as shown in Fig. 2B.

Discussion

Similarly to other MYC family members, c-*MYC* and *MYCL*, the *MYCN* gene is frequently amplified in many types of human cancers including neuroblastoma, and its overexpression is significantly associated with aggressiveness of the tumors [1,27]. In

neuroblastoma, *MYCN* is amplified and closely linked to poor survival probability of patients [4–6]. However, expression levels of *MYCN* in individual tumors are regulated by not only the gene copy number but also the transcriptional regulation [28,29]. Indeed, *MYCN* mRNA is expressed at significantly high levels even in some subsets of neuroblastoma with a single copy number of *MYCN* gene. In the present study, we have demonstrated that MYCN protein enhances its own promoter activity through direct binding to the intron 1 region. To our knowledge, this is the first evidence that MYCN forms a positive auto-regulatory loop through its own transcriptional activation. Since the amplified genomic DNA at *MYCN* gene contains the responsive MYCN binding sites (within intron 1), the identified mechanism may explain the reason why transcription of each *MYCN* gene is activated both in *MYCN*-amplified and non-amplified neuroblastoma cells.

In a sharp contrast to *c-MYC*, the expression of *MYCN* is strictly restricted in both human and mouse adult tissues [9,19,30]. To delineate the essential region of *MYCN* promoter for the tissue-specific expression, the previous studies using transgenic mice showed that the human transgene containing 3.5 kb of upstream and 3 kb of downstream sequences were, at least in part, responsible for the expression pattern of the endogenous *MYCN* gene [31,32]. Further studies revealed that the downstream region including exon 1 and intron 1 is required for tissue-specific promoter activity of *MYCN*, whereas the upstream region regulates basal promoter activity [19]. In support with this notion, the latter region includes a sequence with high homology to the second promoter of *c-MYC*. Furthermore, E2F family proteins bind to enhance basal transcription activity through these regions in both of the two genes [19,20]. Our present results have also revealed that ectopically expressed MYCN activates its own promoter activity through the intron 1 region but not the upstream region, suggesting that the positive auto-regulation of *MYCN* may contribute to maintain tissue-specific expression of *MYCN* rather than basal transcription of the gene.

We have previously found that endogenous expression level of Bcl-2 is one of the keys to determine responsiveness to ATRA for inducing neuronal differentiation or apoptosis in neuroblastoma cells [33]. Indeed, ATRA treatment repressed *MYCN* expression and thereby cells underwent differentiation or apoptotic cell death [12,19]. However, it has been shown that *MYCN* mRNA is transiently down-regulated in response to RA and begins to increase 3–4 days after the administration of RA in several RA-resistant neuroblastoma cell lines [34 and our unpublished observations]. Therefore, it is likely that there could exist at least two distinct molecular mechanisms behind *MYCN* expression in response to RA. The first one is that *MYCN* expression is rapidly down-regulated in response to RA in RA-sensitive neuroblastoma cells. The second one is that RA-mediated repression of *MYCN* is recovered in RA-resistant neuroblastoma cells. Considering that MYCN has an ability to transactivate *MYCN* gene, it is conceivable that this positive auto-regulatory mechanism of *MYCN* expression might be at least in part involved in both cases. Thus, the disruption of this positive auto-regulatory mechanism of *MYCN* expression might provide a novel strategy for developing anti-cancer treatment. To date, it remains unclear how ATRA treatment could cause the down-regulation of *MYCN* in ATRA-sensitive neuroblastoma cells. Further studies should be required to address this issue.

Acknowledgments

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High expression of *ncRAN*, a novel non-coding RNA mapped to chromosome 17q25.1, is associated with poor prognosis in neuroblastoma

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Abstract. Neuroblastoma shows complex patterns of genetic aberrations including *MYCN* amplification, deletion of chromosome 1p or 11q, and gain of chromosome 17q. The 17q gain is frequently observed in high-risk neuroblastomas, however, the candidate genes still remain elusive. In the present study, we integrated the data of comparative genomic hybridization of 236 tumors by BAC array and expression profiling of 136 tumors by using the in-house cDNA microarray carrying 5,340 genes derived from primary neuroblastomas. A novel candidate gene mapped to chromosome 17q25.1 with two splicing variants, *Nbla10727* and *Nbla12061*, was identified. The transcript size appeared to be 2.3 kb by Northern blot, however, the cDNA sequences had no obvious open reading frame. The protein product was undetectable by both *in vivo* and *in vitro* translation assays, suggesting that the transcript might not encode any protein product. Therefore, we named it as *ncRAN* (non-coding RNA expressed in aggressive neuroblastoma). In analysis of 70 patients with sporadic neuroblastoma, the high levels of *ncRAN* mRNA expression were significantly associated with poor outcome of the patients ($p<0.001$). The multivariate analysis showed that expression of *ncRAN* mRNA was an independent prognostic factor among age, stage, origin and *MYCN* expression. Ectopic expression of *ncRAN* induced transformation of NIH3T3 cells in soft agar, while knockdown of endogenous *ncRAN* with RNA interference significantly inhibited cell growth in SH-SY5Y cells. Collectively, our results suggest that *ncRAN* may be a novel non-coding RNA mapped to the region of 17q gain and act like an oncogene in aggressive neuroblastomas.

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Introduction

Neuroblastoma is one of the most common pediatric solid tumors in children and originates from sympathoadrenal lineage of the neural crest. Its clinical behavior is heterogeneous because the tumors often regress spontaneously when developed in patients under one year of age, while they grow rapidly and cause very poor clinical outcome when occurring in patients over one year of age (1). Recent cytogenetic analyses have revealed that given subsets of neuroblastoma with unfavorable prognosis often have *MYCN* amplification, gains of chromosome 1q, 2p, and 17q as well as allelic losses of 1p, 3p, and 11q (1). However, the precise molecular mechanisms underlying pathogenesis and progression of neuroblastoma still remain unclear.

Accumulating evidence shows that gain of chromosome 17 or 17q is the most frequent genetic abnormality in neuroblastoma (1-4). We have previously conducted microarray-based comparative genomic hybridization (array-CGH) with a DNA chip carrying 2,464 BAC clones to examine genomic aberrations in 236 primary neuroblastomas (5). Our array-CGH analysis demonstrated three major groups of genomic aberrations in sporadic neuroblastomas (n=112) that can well define the prognoses of neuroblastomas: a genetic group of silent chromosomal aberration (GGS, 5-year cumulative survival rate: 68%), a genetic group of partial chromosomal gains and/or losses (GGP, 43%), and a genetic group of whole chromosomal gains and/or losses (GGW, 80%). The classification of three genetic groups corresponded well with the pattern of chromosome 17 abnormalities, namely, no gain of either chromosome 17 or 17q, gain of chromosome

17q, and gain of whole chromosome 17, respectively (5). Thus, 17q gain has been implicated in close correlation with aggressiveness of neuroblastoma (5-7). The region has been narrowed down to 17q21-qter, in which several important candidate genes such as *Survivin* and *PPM1D* were suggested to be involved in acquiring aggressiveness of neuroblastoma (4,7,8).

In the present study, by combining with our previous array-CGH data, we searched for the candidate 17q gain gene(s) by applying the results of our gene-expression profiling obtained from the analysis of 136 neuroblastoma samples using an in-house cDNA microarray carrying 5,340 genes isolated from primary neuroblastomas (9,10). This approach has led us to identify a novel non-coding RNA as the candidate mapped to the region of chromosome 17q gain. Its high expression is significantly associated with aggressiveness of primary neuroblastomas.

Materials and methods

Patients. Tumor specimens were collected from the patients with neuroblastoma who had undergone biopsy or surgery at various institutions in Japan. Two hundred and thirty-six and 136 tumor samples were used for array-CGH and expression profiling, respectively (5,10). Among them, sporadic cases were 112 and 70, respectively. The clinical stage of tumor was classified according to the INSS criteria (11). Expression data for the latter 70 sporadic neuroblastomas, which were composed of 15 stage 1, 8 stage 2, 17 stage 3, 25 stage 4, and 5 stage 4s tumors, were used for the Kaplan-Meier analysis. The status of *MYCN* amplification in each tumor had been determined as described previously (8). Patients were treated according to previously described protocols (12,13). The procedure of this study was approved by the Institutional Review Board of the Chiba Cancer Center (CCC19-9).

Microarray-based comparative genomic hybridization (array-CGH) and gene expression profiling. Array-based CGH experiments for 236 neuroblastomas by using a chip carrying 2,464 BAC clones which covers the whole human genome at ~1.2-Mb resolution were performed as described previously (5). For the gene expression profiling of 136 neuroblastomas, we employed an in-house cDNA microarray, carrying 5,340 cDNAs obtained from the oligo-capping cDNA libraries generated from anonymous neuroblastoma tissues (10,14-16). The array-CGH and gene expression profile data are available at NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE 5784 and GSE 5779, respectively.

Cells, culture and transfection. NIH3T3, COS7 and human neuroblastoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 medium containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) and antibiotics. Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. COS7 and NIH3T3 cell lines were transiently transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Construction of expression plasmid. The full-length cDNAs of *Nbla10727* and *Nbla12061* were cloned from the established full length-enriched cDNA libraries which we made from the primary neuroblastomas as described (14-16). The full-length cDNAs were then inserted into pcDNA3 or pcDNA3-FLAG plasmids.

In vitro transcription and translation assay. In vitro translation was carried out in the presence of [^{35}S]-methionine using TNT T7 Quick coupled transcription/translation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The products were resolved by SDS-PAGE and detected by autoradiography.

In vivo [^{35}S]-labeling experiment. COS7 cells were transfected with the FLAG-tagged *ncRAN* expression vectors or the HA-tagged MEL1 expression plasmid. After 24 h, cells were rinsed with 1X PBS 3 times and recultured in fresh growth medium without methionine and antibiotics. Two hours later, [^{35}S]-methionine (GE Healthcare, Tokyo, Japan) was added to the medium to a final concentration of 0.1 mCi/ml, and cells were further incubated. Cells were harvested and whole cell lysates were subjected to immunoprecipitation using a monoclonal anti-Flag antibody or a polyclonal anti-HA antibody. Immunoprecipitates were resolved by SDS-PAGE and detected by autoradiograph.

RNA isolation and semi-quantitative reverse transcription-PCR (RT-PCR). Total RNA was isolated from frozen tumor tissues by an AGPC method (8). Total RNA (5 μg) was employed to synthesize the first-strand cDNA by means of random primers and SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. We prepared appropriate dilutions of each single stranded cDNA for subsequent PCR by monitoring an amount of glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* as a quantitative control. The PCR amplification was carried out for 28 cycles (preheat at 95°C for 2 min, denature at 95°C for 15 sec, annealing at 55°C 15 sec, and extension at 72°C 20 sec) for *ncRAN* (*Nbla10727* and *Nbla12061*). The primers used were: *ncRAN* (*Nbla10727*) 5'-CAGTCAGCCTCAGTTTC CAA-3' (forward); 5'-AGGCAGGGCTGTGCTGAT-3' (reverse), *ncRAN* (*Nbla12061*) 5'-ATGTTAGCTCCCA GCGATGC-3' (forward); 5'-CTAACTGCCAAAAGGTTT TCC-3' (reverse).

Northern blot analysis. Total RNA (20 μg) was subjected to electrophoresis and Northern blotting. The cDNA insert (*Nbla10727*) was labeled with [α-^{32}P]-dCTP (GE Healthcare) by the BcaBEST™ labeling kit (Takara, Tokyo, Japan) and used for the hybridization probe.

Soft agar assay. NIH3T3 cells were transfected with FLAG-*Nbla10727*, FLAG-*Nbla12061* or empty vector, and resuspended in 0.33% agar (wt/vol) in DMEM with 10% FBS at a density of 500 cells/plate. Cell suspensions were poured on the top of the base layer (0.5% agar (wt/vol) in fresh medium, and grew in a 5% CO_2 incubator for 14 days. Colonies >100 μm were counted under an Olympus microscope.

Table I. The comparison of *ncRAN/Nbla10727/Nbla12061* expression level among three major groups of genomic aberrations in neuroblastomas.

Genetic group	n	*ncRAN* expression Mean ± SD (log2 ratio)	p-value	
ncRAN-long/Nbla10727				
GGS (silent)	n=10	-1.12±0.39	p=0.004	
GGP (partial 17q+)	n=26	-0.60±0.48		p=0.952
GGW (whole 17+)	n=35	-1.11±0.48	p<0.001	
ncRAN-short/Nbla12061				
GGS (silent)	n=10	-1.60±0.33	p=0.070	
GGP (partial 17q+)	n=26	-1.23±0.59		p=0.163
GGW (whole 17+)	n=35	-1.81±0.43	p<0.001	

n, number of samples; GGS, Genetic group silent (normal 17); GGP, Genetic group partial gains/losses (17q gain); GGW, Genetic group whole gains/losses (17 gain); *ncRAN* expression levels are shown as normalized log2 ratio of microarray data. p-values were calculated based on statistical t-test.

RNA interference. Oligonucleotides for knocking down the *ncRAN* with *Sac*I and *Xho*I extension were inserted into pMuni vector. The oligonucleotides used were: 5'-CCC CATCCTCTAGTAGCCACGGTTTCAAGAGAACCGT GGCTACTAGAGGATTTTTTGGAAAC-3' and 5'-TCG AGTTTCCAAAAAATCCTCTAGTAGCCACGGTTCTCT TGAAACCGTGGCTACTAGAGGATGGGGAGCT-3'. The plasmids containing the oligonucleotide sequence were transfected into SH-SY5Y cells by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Statistical analysis. The Student's t-tests were used to explore possible associations between *ncRAN* expression and other factors, such as age. Kaplan-Meier curves were calculated and survival distributions were compared using the log-rank test. Univariate and multivariate analyses were made according to the Cox hazard models. q-value was also calculated because *ncRAN* expression was measured with 5340 genes in the microarray (17). Statistical significance was set at $p<0.05$.

Results

Identification of a novel Nbla10727/12061 gene mapped to chromosome 17q25.1 upregulated in advanced neuroblastomas with gain of chromosome 17q. To explore the candidate genes for therapeutic target against aggressive neuroblastomas, the genomic and molecular characteristics specific to high-risk tumors were surveyed. We previously conducted array-CGH analysis with a microarray carrying 2,464 BAC clones to examine genomic aberrations in 236 primary neuroblastomas and found that the gain of chromosome 17q was most strongly correlated with the patient's prognosis (5). The genetic group of 'silent chromosomal aberrations' (GGS) could be defined as the tumor group without apparent abnormalities in chromosome 17, and the genetic group of 'whole chromosomal gains and/or losses' (GGW) as that with gain of whole chromosome 17 (5-year cumulative survival rate in 112 sporadic neuroblastomas: 68 and 80%, respectively, according to ref. 5). On the other hand, the genetic group of 'partial chromosomal gains and/or losses' (GGP) with gain of chromosome 17q showed poor prognosis (43%).

According to the different grade of aggressiveness among the genetic groups, we hypothesized that the GGP tumors may have higher levels of expression of the activated 17q candidate gene(s) that is (are) involved in defining the grade of malignancy of neuroblastoma than the GGS or GGW tumors. We then used our data set of gene expression profile in 136 neuroblastomas to subtract the genes mapped to the commonly gained region of chromosome 17q and differentially expressed in the GGP tumors at high levels and the GGS or GGW tumors at low levels. Consequently, we found two cDNA clones *Nbla10727* and *Nbla12061* (Fig. 1A) on our in-house microarray carrying 5,340 cDNAs obtained from oligo-capping cDNA libraries generated from different subsets of primary neuroblastomas (10,14-16), both of which were splicing variants of the same gene mapped to chromosome 17q25.1 (Table I and Fig. 1B, expression in GGP more than that in GGS or GGW). Database searching showed that both 2,087-bp and 2,186-bp insert sequences (Genbank/DDBJ accession numbers: AB447886 and AB447887) did not exhibit significant similarity to any previously known genes. As the size of mRNA was ~2.3 kb by Northern blot (Fig. 1C), the clones *Nbla10727* and *Nbla12061* appeared to be almost full-length cDNAs. Therefore, *Nbla10727*/12061 appeared to be the gene activated for its expression in neuroblastomas with partial gain of chromosome 17q, but not activated in those with diploid or triploid pattern of whole chromosome 17.

The *Nbla10727*/12061 gene was expressed in multiple human tissues with preferential expression in heart, kidney, lung, spleen, mammary gland, prostate and liver, but with low expression in neuronal tissues such as brain and cerebellum, fetal brain and adrenal gland (Fig. 1D).

High expression of Nbla10727/12061 is associated with poor prognosis of neuroblastoma. The analysis by semi-quantitative RT-PCR in a panel of cDNAs obtained from 8 favorable (stage 1, <1-year-old, single copy of *MYCN* and high expression