いたが，横紋筋肉腫様腫瘍の予後が不良なことが明らかになりNWTS治療研究から除外されている．Wilms腫瘍リスク分類（表1）およびNWTS病期分類（表2）が国際的に用いられている．

NWTS-StageⅣ（StageⅡ～Ⅳ）では，パルス強化療法〔pulse-intensive（single-dose）regimen〕による2年無病生存率は89.4%であり，標準的療法〔standard（divided-dose）regimen〕による90.5%と変わりがなくよい成績であった[1]．このため治療期間の短いパルス強化療法を標準的治療法としてNWTS-Vでは採用し，それに準じてわが国でも日本Wilms腫瘍スタディグループが治療研究を開始している．
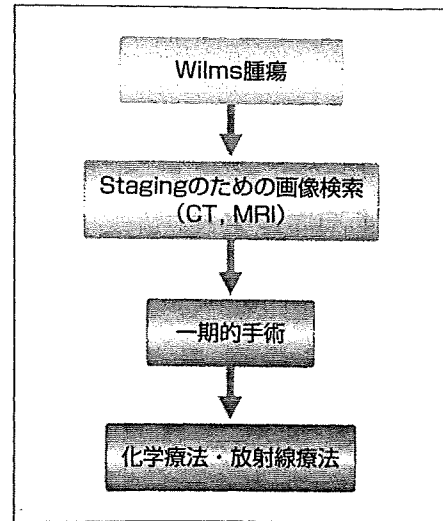

図1　Wilms腫瘍の治療戦略

1 治療戦略

一期的手術の後に，術後化学療法および術後照射が必要となる．術後照射が必要になる症例は，予後良好群のStageⅢ～Ⅳ，退形成腫瘍のStageⅡ～Ⅳ，腎明細胞肉腫の全病期，横紋筋肉腫様腫瘍のStageⅢ～Ⅳである（図1，表3）．

表3　Wilms腫瘍の病期別治療方法（NWTS-V）

病期と年齢・予後因子	治療方法		
	手　術	術後照射	術後化学療法
Stage I/予後良好群 24ヵ月以下，腫瘍550g以下	手　術	な　し	な　し
Stage I/予後良好群 24ヵ月以上，腫瘍550g以上		な　し	EE-4A：ACT-D+VCR（18週）
Stage I/未分化型 （focal of diffuse）		な　し	EE-4A：ACT-D+VCR（18週）
Stage II/予後良好群		な　し	EE-4A：ACT-D+VCR（18週）
Stage III/予後良好群 Stage II-III/未分化型（focal）		10.8 Gy	DD-4A：ACT-D+VCR+DOX（24週）
Stage IV/予後良好群 Stage IV/未分化型（focal）		10.8 Gy	DD-4A：ACT-D+VCR+DOX（24週）
Stage I-III/腎臓明細胞肉腫		10.8 Gy	I：ACT-D+VCR+CPA+VP-16（24週）
Stage II-IV/未分化型（diffuse）		10.8 Gy	I：ACT-D+VCR+CPA+VP-16（24週）

レジメン名	薬剤	用量	スケジュール	
EE-4A	ACT-D	0.045 mg/kg/回	0, 3, 6, 9, 12, 15, 18週	day5から
	VCR	0.05 mg/kg	毎週（10回）	day7から
	VCR	0.067 mg/kg	12, 15, 18週	ACT-D同時投与
DD-4A	ACT-D	0.045 mg/kg/回	0, 3, 6, 9, 12, 15, 18週	day5から
	VCR	0.05 mg/kg	毎週（10回）	day7から
	VCR	0.067 mg/kg	12, 15, 18, 21, 24週	ACT-D, DOX同時投与
	DOX	1.5 mg/kg	3, 9週	
	DOX	1.0 mg/kg	15, 21週	
I	VCR	0.05 mg/kg	1, 2, 4, 5, 6, 7, 8, 10, 11週	day7から
	VCR	0.067 mg/kg	12, 18, 24週	DOX同時投与
	DOX	1.5 mg/kg	0, 6, 12, 18, 24週	
	CPA	14.7 mg/kg/日×3日間	6, 12, 18, 24週	
	CPA	14.7 mg/kg/日×5日間	3, 9, 15, 21週	
	VP-16	3.3 mg/kg/日×5日間	3, 9, 15, 21週	

ACT-D：アクチノマイシンD，VCR：ビンクリスチン，DOX：ドキソルビシン，CPA：シクロホスファミド，VP-16：エトポシド

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手術日を術後0日とすると,化学療法は術後5日から開始し,術後照射開始も術後9日より遅れてはならない.

2 治療方法 (表3)

術後照射における肉眼的標的体積 (GTV) は,手術前 (初診時) CTにて認められた原発巣および所属リンパ節転移巣である.計画標的体積 (PTV) として,外側は側腹壁を照射野に入れ,内側は対側傍大動脈リンパ節領域を含み,側弯症予防のためにも椎体骨全幅が照射野に入るような前後二門照射野とする (図2).

線量は,治療線量10.8 Gy/6回,週5日間照射を原則とする (表3).腹腔全体の腫瘍漏出や腫瘍播種が認められた時は,全腹部照射 (横隔膜ドームから閉鎖孔まで.大腿骨頭は遮蔽) とし,1日線量1.5 Gy,総線量10.5 Gyとする.しかし残存腫瘍が大きく局所再発をきたす可能性の高い時には,追加照射10.8 Gyが必要である.

図2 Wilms腫瘍の術後照射野シミュレーション

一期的切除術にて全摘したstageⅢ右腎原発Wilms腫瘍に対する術後照射シミュレーション.初診時腫瘍を含む腎臓全体および椎体骨全幅が,照射野に含まれていることが必要である.

リスク臓器として,残存腎は鉛ブロックで遮蔽するか,1/3以上は14.4 Gy/8回を超えないようにし,また正常肝臓の1/2以上へは19.8 Gy/11回を超えないようにする.18ヵ月以下の乳幼児に対しては化学療法を用い,放射線治療は控える.

また,術後早期に化学療法を開始することが必要である.手術日をday0として,day5から予後良好群ではEE-4A,DD-4Aを開始することとなっている (表3).骨髄抑制として白血球減少が8〜14日に起こってくるが,化学療法はできるだけ各クールの開始時期を遅らせないようにする必要がある.シクロホスファミドによる出血性膀胱炎を予防するため,メスナが用いられる.

3 副作用対策

NWTSにおいて,臓器・組織耐容線量を考慮した照射線量が規定されているので,それを遵守する限りにおいて放射線晩期合併症 (副作用) は問題とはならない.

2. 神経芽腫の治療

神経芽腫は,交感神経系組織が存在する副腎髄質または傍脊髄部を原発巣とし,幼児期に多く,年間約150症例発生する.

神経芽腫の発生部位は副腎が最も多く,その他は頸部,後縦隔,後腹膜,骨盤腔などの交感神経節である.腹部膨満,顔面蒼白,貧血,食欲不振などを初発症状とし,遠隔転移症状としての眼球突出,眼瞼出血,跛行,骨痛などで発見されることが多い.

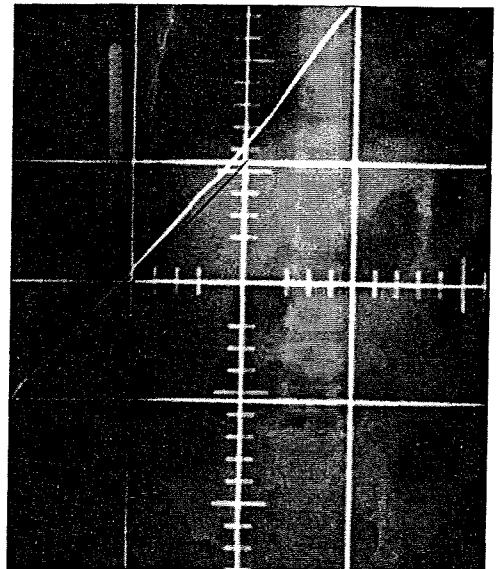神経芽腫はカテコールアミン産生腫瘍であり,その代謝産物としての尿中バニルマンデル酸 (vanilmandelic acid：VMA) およびホモバニリン酸 (homovanilic acid：HVA) が腫瘍マーカーとして

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用いられている.

　小児固形腫瘍で最も多い神経芽腫は予後不良とされていたが，6ヵ月乳児検診時のマススクリーニングにて早期症例が発見されるようになり，1歳以下のマススクリーニング症例ではほとんど治癒するようになった．しかし，この中には自然消腿するものが含まれており，過剰な治療が施されているという欧米の論文が散見されるようになり，マススクリーニングは中止となった.

　間質増生量，神経芽細胞の成熟度および神経芽細胞の核分裂-核崩壊指数の組織学的パラメーターと患者の年齢に基づいて，予後良好か不良かを明らかにしている臨床病理学的Shimada分類が用いられ，腫瘍細胞の染色体数が2倍体，N-myc遺伝子の増幅，trkA遺伝子の低発現，血清（neuron specific enolase：NSE）の高値が予後不良因子となっている．これらの予後不良因子のない群には治療軽減が考えられているが，予後不良因子をもつ進行症例には骨髄破壊的大量化学療法を採用している.

　Stagingは，神経芽腫国際病期分類（International Neuroblastoma Staging System：INSS）が世界的に認知されており，^{123}I MIBG（metaiodo-benzylguanidine）シンチグラフィが原発巣および転移巣の把握，さらに治療経過観察に必須とされている（表4，図3）.

表4　神経芽腫国際病期分類（INSS）

1期	限局性腫瘍で，肉眼的に完全切除．組織学的な腫瘍残存は不問．同側のリンパ節に組織学的な転移を認めない（原発腫瘍に接し，一緒に切除されたリンパ節転移はあってもよい）.
2A期	限局性腫瘍で，肉眼的に不完全切除．原発腫瘍に接しない同側リンパ節に組織学的に転移を認めない.
2B期	限局性腫瘍で，肉眼的に完全または不完全切除．原発腫瘍に接しない同側リンパ節に組織学的に転移を認める．対側のリンパ節に転移を認めない.
3期	切除不能の片側性腫瘍で，正中線（対側椎体縁）を越えて浸潤．同側の局所リンパ節の転移は不問．または，片側発生の限局性腫瘍で対側リンパ節転移を認める．または，正中発生の腫瘍で椎体縁を越えた両側浸潤（切除不能）か，両側リンパ節転移を認める.
4期	いかなる原発腫瘍であるかにかかわらず，遠隔リンパ節，及び/または，骨，骨髄，肝，皮膚，他の臓器に播種している（4Sは除く）.
4S期	限局性腫瘍（病期1，2A，2B）で，播種は皮膚，及び/または，肝，骨髄に限られる（1歳未満の患者のみ）．骨髄中の腫瘍細胞は有核細胞の10％未満で，それ以上は病期4である．MIBGシンチが行われるならば骨髄への集積は陰性.

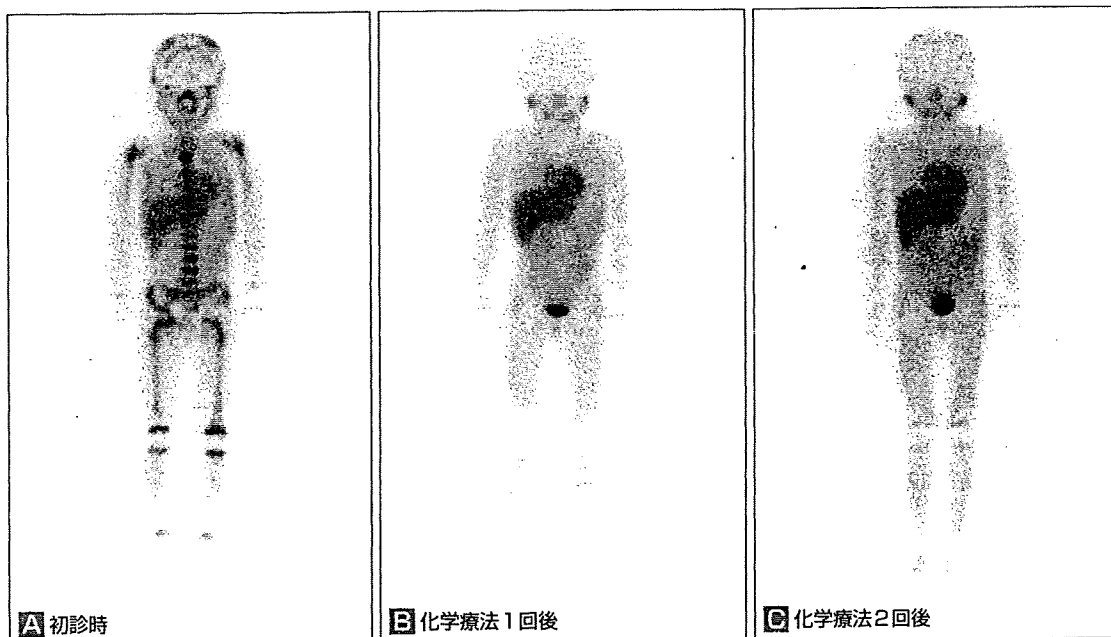Ⓐ 初診時　　Ⓑ 化学療法1回後　　Ⓒ 化学療法2回後

図3　神経芽腫5歳女児　左副腎原発Stage 4のMIBGシンチグラフィ

^{123}I MIBGシンチグラフィにて原発巣および全身骨格に集積を認めたが（Ⓐ），左腸骨，右恥骨部は単純写真にて骨皮質転移が証明され，骨盤骨以外の全身骨格系への集積は骨髄転移と考えられている．化学療法により集積は陰性化し（Ⓑ，Ⓒ），チオテパ＋メルファランの前処置で自家末梢血幹細胞移植施行した．その後，遅延一期的切除と術後照射（19.8 Gy/11回）が行われた.

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📖 **用語解説**

• ^{123}I MIBGシンチグラフィ：神経芽腫の特異的診断法とされている神経芽腫は，カテコールアミンを産生する functional tumorである．^{123}I metaiodo-benzylguanidine（MIBG）は，交感神経遮断薬であるguanidineのアナログであり，ノルエピネフリン再摂取機構を介し，ノルエピネフリン貯蔵顆粒に取り込まれるとされている．

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1 治療戦略(図4)

現在，一般的に行われている進行神経芽腫の治療戦略は，寛解導入療法としての化学療法（neo-adjuvant）を4～5コース行った後，局所療法として二期的手術および局所放射線療法を行い，その後強化した化学療法あるいは骨髄破壊的大量化学療法による地固め療法（adjuvant）を行う．

導入化学療法による血液毒性は強いが，普通は血球減少により放射線治療を中断する必要はない．血球数に問題がある場合にも，放射線治療が完遂するまで，小児腫瘍医の判断を尊重すべきである．

2 治療方法

肉眼的標的体積（GTV）は，手術前（初診時）CTにて認められた原発巣およびリンパ節転移巣すべてを含む．

1回線量1.8 Gyで，週5日間照射を原則とする．

導入化学療法後の遅延一期的切除（delayed primary surgery）あるいは二期的手術が主流で，骨髄破壊的化学療法が行われる現在，CCG-3891（Children's Cancer Group）研究では，シスプラチン60 mg/m^2（day1），ドキソルビシン30 mg/m^2（day3），エトポシド100 mg/m^2（day3～6），シクロホスファミド1,000 mg/m^2（day4, 5）の組み合わせによる寛解導入療法を28日ごとに5サイクルを行う方法により縮小した腫瘍巣をGTVとし，術後照射線量20 Gyを採用している．骨転移部も化学療法にあわせての20 Gyにて制御可能である[2]．
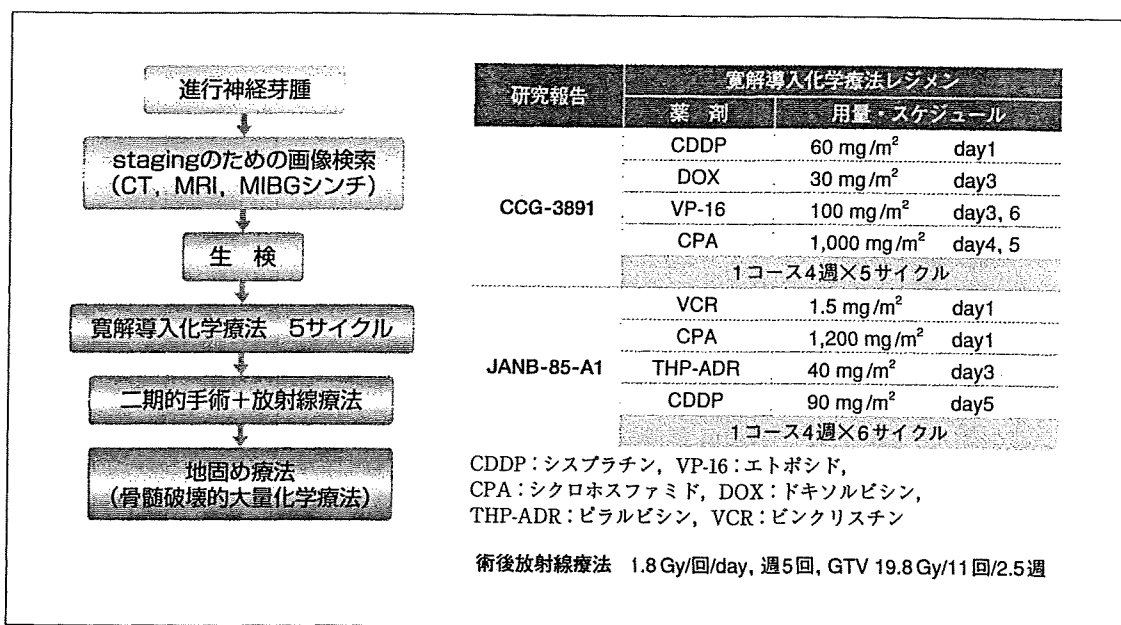
この先行研究として，1985年に澤口が寛解導入療法（JANB-85-A1）を世界に先駆けて開発し，シ

研究報告	寛解導入化学療法レジメン		
	薬剤	用量・スケジュール	
CCG-3891	CDDP	60 mg/m^2	day1
	DOX	30 mg/m^2	day3
	VP-16	100 mg/m^2	day3, 6
	CPA	1,000 mg/m^2	day4, 5
	1コース4週×5サイクル		
JANB-85-A1	VCR	1.5 mg/m^2	day1
	CPA	1,200 mg/m^2	day1
	THP-ADR	40 mg/m^2	day3
	CDDP	90 mg/m^2	day5
	1コース4週×6サイクル		

CDDP：シスプラチン，VP-16：エトポシド，
CPA：シクロホスファミド，DOX：ドキソルビシン，
THP-ADR：ピラルビシン，VCR：ビンクリスチン

術後放射線療法 1.8 Gy/回/day，週5回，GTV 19.8 Gy/11回/2.5週

図4　進行神経芽腫の治療戦略

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クロホスファミド1,200 mg/m^2, ビンクリスチン1.5 mg/m^2, ピラルビシン40 mg/m^2, シスプラチン90 mg/m^{2}という寛解導入化学療法を28日ごとに6サイクル行うことにより，治療成績の向上が認められた[3]（図4）.

3 大量化学療法と自家造血幹細胞移植

全身的な微小転移巣，特に骨・骨髄転移の根絶を期待して，すなわちtotal cell killとして大量化学療法を行うが，その際に造血幹細胞サルベージ療法としての造血幹細胞移植があり，自家移植と同種移植とがある．自家移植に用いる造血幹細胞は，骨髄あるいは末梢血幹細胞から採取している．同種移植においては，ドナー由来の免疫担当細胞がレシピエント体内に残存するがん細胞に対して免疫反応を起こす「同種免疫反応による抗腫瘍効果」も期待できる.

CCG-3891研究において，**骨髄破壊的大量化学療法群**と強化した非骨髄破壊的化学療法群の比較が行われ，非骨髄破壊的化学療法群の3年無イベント生存期間（event-free survival；EFS）が22±4％であったのに対し，骨髄破壊的大量化学療法群では34±4％と有意に優れていた[2]．これらの結果より，後続の臨床研究では，進行神経芽腫において骨髄破壊的大量化学療法が地固め療法として採用されている.

> **用語解説**
>
> ● **骨髄破壊的大量化学療法による地固め療法**：導入化学療法などの従来の化学療法では，骨髄障害のために，がん細胞を根絶するまでの化学療法を強化できなかった．この骨髄障害を問題としない，total cell killを狙った地固め療法として，造血幹細胞移植を前提とした大量化学療法が開発された.
>
> ● **大量化学療法の代表的なレジメン**
> 例1）チオテパ(TEPA) 200 mg/m^2/day＋メルファラン(L-PAM) 70 mg/m^2/day
> 例2）MEC療法
>
> | | 1回量 | 総投与量 |
> |---|---|---|
> | メルファラン(L-PAM) | 100 mg/m^2 | 200 mg/m^2 |
> | エトポシド(VP-16) | 200 mg/m^2 | 800 mg/m^2 |
> | カルボプラチン(CBDCA) | 400 mg/m^2 | 1,600 mg/m^2 |
>
> このほかには，シスプラチン＋メルファラン＋エトポシド，ブスルファン＋メルファラン，メルファラン＋エトポシド＋カルボプラチン＋ドキソルビシン，などがある.

4 副作用対策

骨発育障害は6〜10 Gyで現れ20 Gyで明らかとなるため，照射野設定の際，なるべく骨端線を含まないようにする.

3. 横紋筋肉腫の治療

横紋筋肉腫は局所的に浸潤，進展する腫瘍であり，手術のみでは局所再発をきたしやすく，早期に遠隔転移を起こしやすい．米国Intergroup Rhabdomyosarcoma Study (IRS)により，手術後の化学療法と放射線治療の有効性が示された.

横紋筋肉腫は，将来骨格筋を形成する胎児の中胚葉，または間葉組織に発生する腫瘍であるが，本来骨格筋のない部位からも発生するもので，年間60〜80例発症している．発生部位により，予後

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が分かれる．予後良好部位は眼窩，頭頸部（傍髄膜を除く），泌尿生殖器（膀胱，前立腺を除く），胆道であり，予後不良部位は膀胱・前立腺，四肢，傍髄膜，ほか（体幹，後腹膜，会陰・肛門周囲，胸腔内，消化管，胆道を除く肝臓）となっている．

組織分類による予後では，胎児型（embryonal type），ぶどう状肉腫型（botryoid type）は予後良好であり，染色体転座による*PAX3/7-FKHR*キメラ遺伝子をもつ胞巣型（alveolar type）は予後不良である．発生部位，腫瘍サイズなどを考慮したIRS-staging systemと，術後診断によるIRS-grouping systemを併せたIRSによる予後分析により，リスク分類が行われるようになった（表5，6，7）．

1 治療戦略

clinical group I胎児型には放射線治療は不要であるが，胞巣型には術後顕微鏡的残存（clinical group II）と同様な術後照射が必要である[4]．肉眼的残存腫瘍（clinical group III）の術後照射線量を

表5 横紋筋肉腫の術前Stage分類（IRS pre-treatment TNM staging classification）

Stage	原発部位（Sites）	原発腫瘍（T）	大きさ（Size）	領域リンパ節（N）	遠隔転移（M）
1	眼窩，頭頸部（傍髄膜を除く），泌尿生殖器（膀胱，前立腺を除く），胆道	T1 or T2	a or b	N0 or N1 or Nx	M0
2	膀胱・前立腺，四肢，傍髄膜 ほか（体幹，後腹膜，会陰・肛門周囲，胸腔内，消化管，胆道を除く肝臓）	T1 or T2	a	N0 or Nx	M0
3	膀胱・前立腺，四肢，傍髄膜 ほか	T1 or T2	a	N1	M0
			b	N1 or N0 or Nx	M0
4	すべて	T1 or T2	a or b	N0 or N1	M1

・原発腫瘍（T）−T1：原発部位に限局，T2：原発部位を越えて進展または周囲組織に癒着
・大きさ（Size）−a：最大径で5 cm以下，b：最大径で5 cmを越える
・領域リンパ節（N）−N0：リンパ節転移なし，N1：領域リンパ節に転移あり（画像または身体的所見上），Nx：転移の有無は不明（特に領域リンパ節転移の評価困難な部位）
・遠隔転移（M）−M0：なし，M1：あり

表6 横紋筋肉腫の術後Group分類（IRS clinical grouping classification）

Clinical Group	I	組織学的に全摘除された限局性腫瘍 　a. 原発臓器または筋に限局 　b. 原発臓器または筋を越えて（筋膜を越えて）周囲に浸潤 ただし，いずれの場合も領域リンパ節に転移は認めない （頭頸部を除いてサンプリングまたは廓清により組織学的確認を必要とする）
	II	肉眼的に全摘除された領域内進展腫瘍 　a. 切除断端に顕微鏡的腫瘍遺残あり，ただし，領域リンパ節に転移を認めない 　b. 領域リンパ節に転移を認めるが完全摘除を行った．即ち，最も遠位の廓清リンパ節に転移を認めない 　c. 領域リンパ節に転移を認め，しかも，切除断端に顕微鏡的腫瘍遺残を認めるが，最も遠位の廓清リンパ節に転移を認める
	III	肉眼的な腫瘍遺残 　a. 生検のみ施行 　b. 亜全摘除または50％以上の部分摘除を施行
	IV	a. 遠隔転移（肺，肝，骨，骨髄，脳，遠隔筋組織，遠隔リンパ節など）を認める b. 脳脊髄液，胸水，腹水中に腫瘍細胞が存在 c. 胸膜播種，腹膜（大網）播種を伴う

表7 横紋筋肉腫のリスク分類（IRS-V）

リスク群	組織型	Stage（表5）	Group（表6）
低リスク群	胎児型	1	I, II, III
	胎児型	2, 3	I, II
中間リスク群	胎児型	2, 3	III
	胞巣型	1, 2, 3	I, II, III
高リスク群	胎児型または胞巣型	4	IV

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導入および術後化学療法：VAC療法（レジメン44）

ビンクリスチン(VCR)：1.5 mg/m² (day1)

アクチノマイシンD(ACT-D)：0.015 mg/kg/day(day1, 2, 3, 4, 5)

シクロホスファミド(CPA)：2.2 g/m² (day1)(メスナ併用)

3週ごと46週行い，8週目と19週目で評価する．

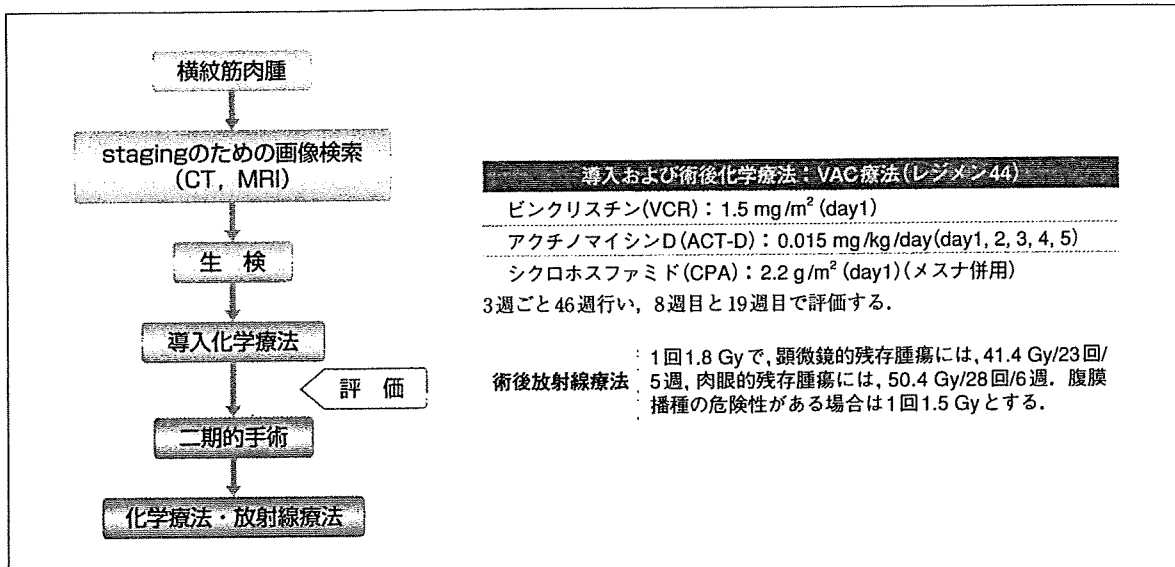術後放射線療法：1回1.8 Gyで，顕微鏡的残存腫瘍には，41.4 Gy/23回/5週，肉眼的残存腫瘍には，50.4 Gy/28回/6週．腹膜播種の危険性がある場合は1回1.5 Gyとする．

図5 横紋筋肉腫の治療戦略

下げるためと機能温存のために，現在の標準的治療であるVAC療法（ビンクリスチン＋アクチノマイシンD＋シクロホスファミド）と，局所療法としての二期的手術および術後放射線治療が必須となっている（図5）．化学療法後の評価により二期的手術適応が検討され，腫瘍全摘を試みる治療研究がIRS-Vおよび日本横紋筋肉腫治療研究グループで進行している．

2 治療方法

GTVは，初診時の理学的所見やCT・MRI所見にて認められた腫瘍巣である．この領域には病理学的に転移の認められたリンパ節だけでなく，すべての腫大したリンパ節領域も含まれる．

顕微鏡的残存腫瘍においては1回線量1.8 Gyで41.4 Gy/23回/5週，肉眼的残存腫瘍において50.4 Gy/28回/6週照射が標準的であるが，腹膜播種の危険性が認められる場合の全腹部照射では1回線量を1.5 Gyと低くする[4]．なお，正常組織耐容線量を超えないように考慮し，照射野漸減法(shrinking field technique)により正常組織の障害を最小限にすることが必要である．

傍髄膜領域あるいは髄膜進展が認められたものに対しては，診断がつき次第，放射線治療を始めなければならない．いわゆるゴールデンタイム（72時間以上完全脊髄機能障害があれば回復の望みがない）を超えなければ，緊急放射線治療にて不可逆的変化をきたさずにすむ．

3 副作用対策

横紋筋肉腫の局所治療として放射線治療を施行する限りにおいて，小児正常組織への影響は免れないものであり，常に合併症を念頭におかねばならない．IRS-Ⅱ，Ⅲでの頭頸部腫瘍（眼窩を除く）研究では，77％に晩期副作用が発生している[5]．

この晩期副作用の代表的なものとして，身長発育不良，頭頸部変形（組織形成不全あるいは非対称性による），不良歯列および異常歯，白内障・角膜変化と眼萎縮による視力障害，聴力障害(cisplatinによる影響もある)，学習障害，二次性悪性腫瘍（肉腫，がん，白血病）が認められている．これに対処するため，成長ホルモン投与，形成外科的再建術，補聴器着用などを配慮すべきである．

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さらに，化学療法・放射線療法併用であることから二次発がんの標準化発生率が5%前後と高いことを理解し，follow-up体制をとる必要がある[6].

小児がん治療における他科との連携のポイント

小児がんの放射線治療においては根治線量を投与できないことが多いので，化学療法の補助療法（adjuvant therapy）ととらえて集学的治療の一翼を担うという考えを持つべきである．したがって，化学療法同時併用となる場合が多いが，骨髄抑制期であっても小児腫瘍医の管理体制を信頼し，split courseを置かず，放射線治療を継続することも重要である．

術後照射ではなるべく術後早期に放射線治療を開始することが必要であり，Wilms腫瘍では術後9日以内に放射線治療開始が要求されている．当然，化学療法も術直後からの開始が必要となっており，これは手術による局所再発および遠隔転移リスクを下げるための重要な因子であることを理解していただきたい．

治療のコツ・ポイント

小児においては，検査であろうと放射線治療であろうと，患児の協力が得られることができれば，ほぼこれが達成できたと言われており，そのための環境作りが重要である．筆者の施設では液晶テレビで好きなアニメ映像を見せることにより，2歳以上であれば無鎮静で放射線治療を行っている．この環境に慣れさせるために，模擬照射として数日間をリニアック室で体験してもらっている．家族への甘えが大きい子供にあっては，病棟出棟時より看護師のみと来室してもらっている．

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（正木英一）

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付表1　抗がん剤の取り扱い上の注意

取り扱い注意度	判定基準	抗がん剤 (化学放射線療法に頻用される薬剤を中心に)	
A	・細胞毒性が強く，その取扱いに十分注意が必要な抗がん剤 (取り扱いに注意を要するもの) 1. 毒薬指定となっているもの 2. 催奇形性または発がん性がヒトで報告されているもの (または疑われているもの) 3. その他，取り扱い上の注意事項でとくに留意すべきもの 4. 上記のいずれかに該当するもの	アルキル化薬	シクロホスファミド イホスファミド ダカルバジン
		白金製剤	シスプラチン カルボプラチン
		抗がん性抗生物質	ドキソルビシン エピルビシン ミトキサントロン マイトマイシンC
		代謝拮抗薬	フルオロウラシル メトトレキサート
		微小管作用薬	パクリタキセル ドセタキセル ビノレルビン
B	・細胞毒性が強く，その取扱いに注意が必要な抗がん剤 (取り扱いにやや注意を要するもの) ・動物による変異原性 (in vitroまたはin vivo)，催奇形性または発がん性のいずれかの実験で陽性所見の報告があるもので，Aに該当しないもの	抗がん性抗生物質	アクラルビシン ブレオマイシン
		代謝拮抗薬	ゲムシタビン シタラビン
		微小管作用薬	ビンクリスチン ビンブラスチン ビンデシン
		トポイソメラーゼ阻害薬	エトポシド イリノテカン
C	・細胞毒性に注意する抗がん剤 (ふつうの薬物と同じ取り扱いで良いもの) ・変異原性，催奇形性，発がん性のいずれもが陰性で，特に毒性が強くないもの	抗体医薬	トラスツズマブ リツキシマブ
		その他	レンチナン 溶連菌抽出物

(日本病院薬剤師会学術委員会編：抗悪性腫瘍剤の院内取扱い指針. P.17〜21, 日本病院薬剤師会, 1991及び日本病院薬剤師会監修：抗悪性腫瘍剤の院内取扱い指針 改訂版 抗がん剤調製マニュアル. P.149〜168, じほう, 2005を参考資料にして作成)

付表2　安全キャビネットが設置されていない施設での抗がん剤取り扱い時における実務対策

〈準　備〉
1. 作業エリアの範囲を決めておく
・作業用シートをきちんと敷ける広さで，調製作業が支障なく行えること
・流し台が近くにあることが望ましい (万一の事故の場合，洗浄ができる流し台)
2. 調製に必要な器具・用具
・ディスポーザブルの作業用シート (裏が撥水性のビニール)
・ディスポーザブルのマスク，手袋
・保護メガネ (眼鏡で代用可能)
・ディスポーザブルのキャップ，ガウン
・ディスポーザブルの注射シリンジ・注射針
・廃棄用容器 (感染性医療廃棄物用プラスチックケースを流用)
・スピル・キット (抗がん剤のスピル事故時に備える)

〈実　施〉
1. 調製時の全般的な注意事項
・抗がん剤に対する知識・作業手順を習得した上で調製を行う
・作業前に必要な薬剤，器具，用具等の不足がないようあらかじめ準備する
・作業エリアは整理・整頓を心がけ，他の薬剤等の作業エリアと区分する
・作業終了時，作業エリアを十分清掃する
・各作業終了後，十分な手洗いとうがいを励行する
2. 調製時の注意事項
・安全キャビネットが設置されていないので，あらかじめ決められた一定の作業エリアで行う
・作業用シートの上で調製を行い，作業環境への汚染には十分留意する
・調製する者は，マスク，保護メガネ，手袋，ガウンを必ず着用する。

保護メガネは，通常の眼鏡でも差し支えない. 調製に慣れていない者は，これらに加え，キャップを着用する.
飛沫汚染があった場合は，すぐに新しいものと交換する
・バイアルタイプの抗がん剤の調製時には，シリンジ針をバイアルより抜く際にエアロゾルが発生する.
このエアロゾルの発生を防ぐためバイアル内を陽圧としないよう注意する
3. 廃棄処理
・抗がん剤の残薬，薬剤の容器，使い捨て器具・用具，清拭に使用した布・紙類，使用した防具などをはじめ，抗がん剤が付着した可能性のあるものは，焼却処理するため，調製エリアにあらかじめ用意した感染性医療廃棄物用プラスチックケース (廃棄用容器として流用) 内に廃棄する
4. その他
・シリンジはルアーロックシリンジが望ましい
・アンプルを割った帽状の方にも薬液が付いているので注意
・補液ビン，補液バックの口のところ (ゴム栓) に素手で触れない (薬液付着あり)
・持続注入器 (LVなど) に詰めるときは，先に溶媒を加えてから次に薬液を加える
・シスプラチン，シタラビンなどの高用量調製時には，点滴ボトルに入りきらないことがあるので，事前に確認する
・ドキソルビシンは液漏れし易いので，細めの注射針を使用する
・シクロホスファミドは溶解し難いので，溶け残りの有無に注意する
・パクリタキセルの溶解液は多目に入っているが全部加えること
・ドセタキセルの非アルコール溶解は溶けにくいので，溶け残りの有無に注意する
・トラスツズマブは溶解時激しく震盪しない (泡立ちやすい)

(日本病院薬剤師会監修：抗悪性腫瘍剤の院内取扱い指針 改訂版 抗がん剤調製マニュアル. P.134 じほう, 2005を参考資料にして作成)

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 knock-down 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.

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

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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
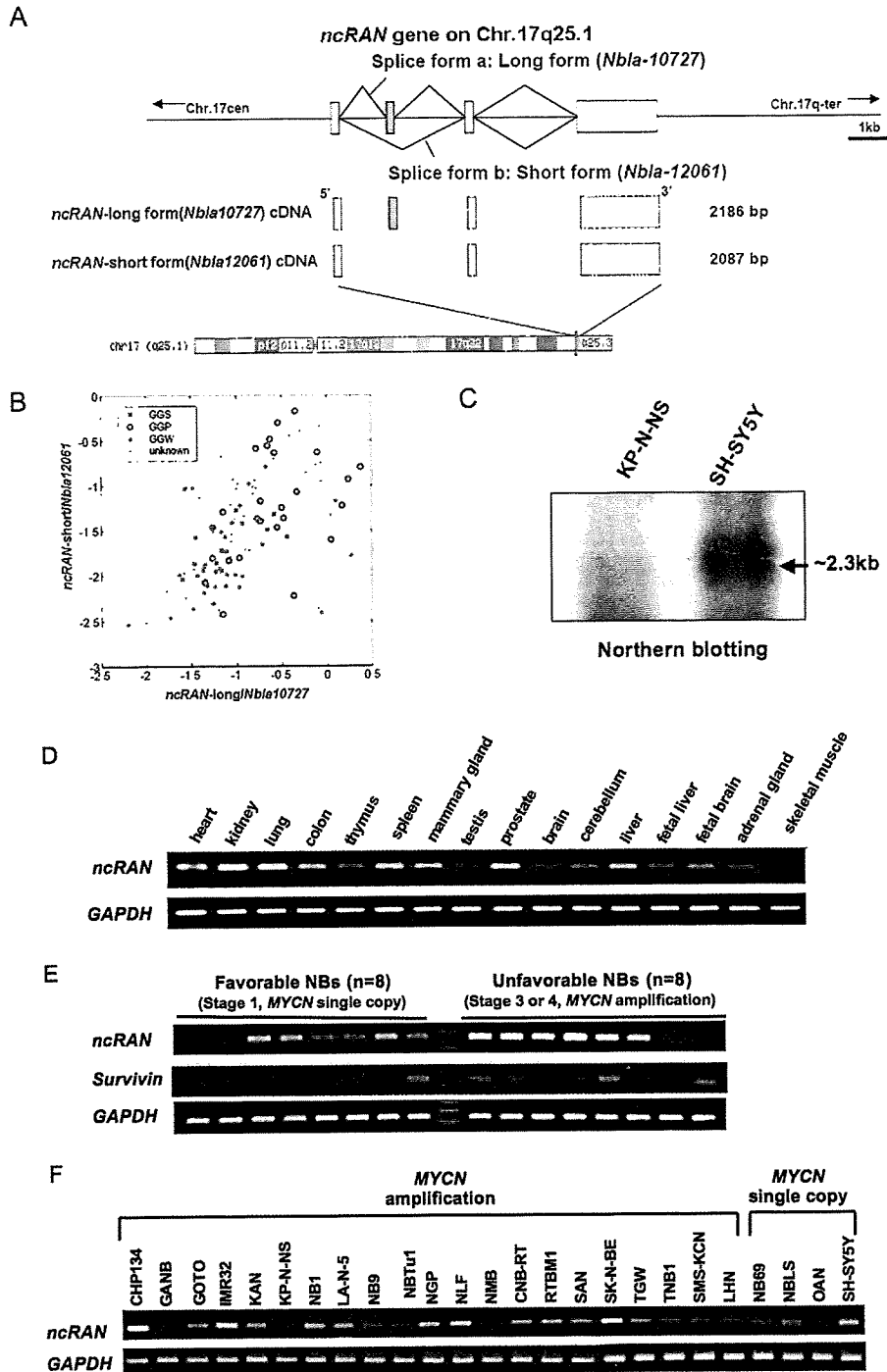
Figure 1. *ncRAN* is mapped to the 17q gain region. A, Genomic structure of *ncRAN* region on chromosome 17q25.1. Splicing variants, whose sequences were contained in cDNAs as *ncRAN-long/Nbla10727* and *ncRAN-short/Nbla12061*, are schematically shown. These are transcribed from a single gene, *ncRAN* (see text). B, High expression of *ncRAN* is associated with high malignant subset of neuroblastoma. Scatter plot of the expression levels of the *ncRAN-long/ Nbla10727* and *ncRAN-short/Nbla12061* in 71 primary neuroblastomas with both accompanying expression and aCGH data. Blue, red, green, and black spots denote GGS, GGP, GGW and unknown genomic group samples, respectively. As shown in Table I, the expression levels of the *ncRAN* were significantly higher in GGP tumors (+17q gain) than in GGS (no 17 gain) or GGW (+ whole 17 gain) tumors (p=0.004 and p<0.001 for *ncRAN-long/Nbla10727*, and p=0.070 and p<0.001 for *ncRAN-short/Nbla12061*, respectively), whereas their expression levels in GGS and GGW tumors were comparable (p=0.952 for *ncRAN-long/Nbla10727*, and p=0.163 for *ncRAN-short/Nbla12061*, see also Table I), suggesting that the acquired allele(s) at 17q might be silenced at least for the *ncRAN* expression in GGW tumors, and that high expression of *ncRAN* is associated with high malignant subset of neuroblastoma. C, Northern blot analysis of *ncRAN*. Total RNA (20 μg) prepared from neuroblastoma cell lines, SH-SY5Y and KP-N-NS were used. A 2.3-kb band was visible in only SH-SY5Y cells. The cDNA insert *(Nbla10727)* was labeled with [α-^{32}P]-dCTP and used for the hybridization probe. D, Semiquantitative RT-PCR of *ncRAN* in multiple human tissues and neuroblastoma cell lines. Total RNA of 25 adult tissues and two fetal tissues were purchased from Clontech Co. Ltd. The expression of *GAPDH* is also shown as a control. E, Semi-quantitative RT-PCR of *ncRAN* in favorable and unfavorable subsets of primary neuroblastomas. The mRNA expression patterns for *ncRAN* and *Survivn*, a known oncogene identified at 17q, were detected by semi-quantitative RT-PCR procedure in eight favorable (lanes: 1-8, stage 1, with a single copy of *MYCN*) and eight unfavorable (lanes: 9-16, stage 3 or 4, with *MYCN* amplification) neuroblastomas. F, Semiquantitative RT-PCR of *ncRAN* in neuroblastoma cell lines. Twenty-one neuroblastoma cell lines with *MYCN* amplification and 4 cell lines with a single copy of *MYCN* were used for this study as templates.
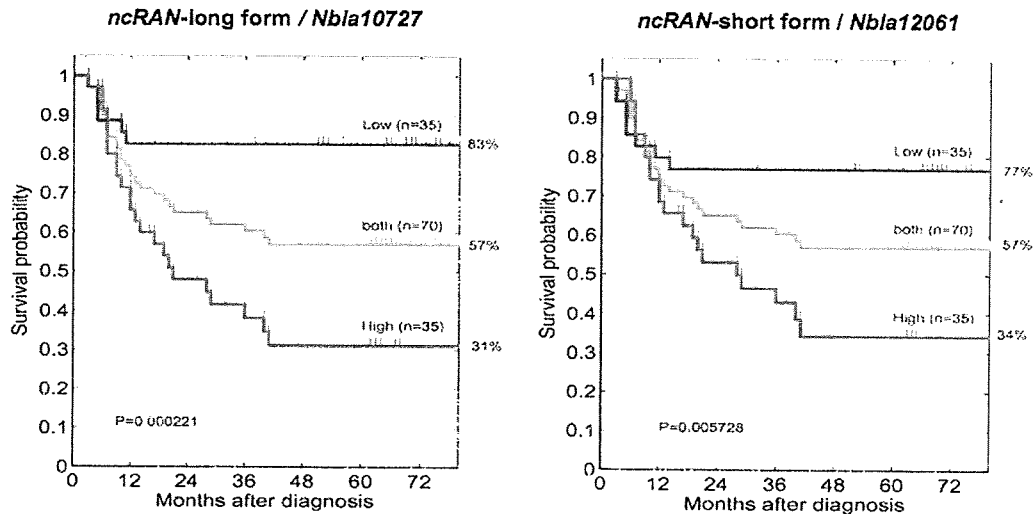
Figure 2. The high expression of *ncRAN/Nbla10727/12061* mRNA is a prognostic indicator of unfavorable neuroblastomas. The Kaplan-Meier survival curves were drawn from the results of the cDNA microarray data of 70 sporadic neuroblastomas (log-rank test, p=0.000221 and p=0.005728, respectively).

of *TrkA*) and 8 unfavorable (stage 3 or 4, >1-year-old, amplified *MYCN* and low expression of *TrkA*) primary neuroblastomas confirmed that this novel gene was expressed at significantly high levels in the latter compared to the former (Fig. 1E), such as *Survivin* which we have previously reported as one of the candidate genes mapped at the region of 17q gain (9). Among neuroblastoma cell lines, high or moderate levels of expression of *Nbla10727/12061* was observed in cell lines with *MYCN* amplification most of which had 17q gain, whereas it was relatively low in those with a single copy of *MYCN* and without the 17q gain (Fig. 1F).

As shown in Fig. 2, our microarray data of 70 sporadic neuroblastomas showed that the high levels of *Nbla10727/ 12061* expression were significantly associated with poor prognosis (log-rank test, p=0.000221 and p=0.005728, respectively). The multivariate analysis using Cox proportional hazard model demonstrated that expression of *Nbla10727/ 12061* was an independent prognostic factor among age at diagnosis, disease stage, tumor origin and *MYCN* expression (Table II). Thus, the expression level of *Nbla10727/12061* is a novel prognostic factor of neuroblastoma that is closely associated with gain of chromosome 17q.

Nbla10727/12061 is involved in inducing enhancement of cell growth in neuroblastoma cells and transformation of NIH3T3 cells. To investigate function of *Nbla10727/12061*, we transfected SH-SY5Y neuroblastoma cells with the siRNA, since SH-SY5Y cells have 17q gain in their genome as well as higher mRNA expression of *Nbla10727/12061*. As shown in Fig. 3A, suppression of endogenous levels of *Nbla10727/ 12061* transcripts significantly inhibited cell growth in SH-SY5Y neuroblastoma cells as compared with the control cells. On the other hand, the soft agar colony formation assay showed that the enforced expression of *Nbla10727/12061* significantly enhanced the anchorage-independent growth of NIH3T3 mouse fibroblast cells (Fig. 3B). These results suggested that *Nbla10727/12061* was a novel candidate gene of the region of 17q gain with an oncogenic function.

ncRAN-Nbla10727/12061 is a large non-coding RNA. Several lines of evidence from the gene structure analysis as well as the comparative genomic analysis described below further suggested that *Nbla10727/12061* is a non protein-coding but functional RNA. We therefore tentatively named this gene as *ncRAN* (non-coding RNA expressed in aggressive neuroblastoma).

First, the full-length cDNA sequences of *ncRAN*, which are suggested to be relevant to both *Nbla10727* and *Nbla12061* cDNAs by Northern blot analysis (Fig. 1C), did not contain any long-enough open reading frames (>200 bp). Bioinformatic analysis indicated that there were no ESTs longer than those two cDNAs at the genomic locus, and that the CpG island was located at the 5' region of the cDNA sequences.

Second, no protein product was translated both *in vivo* and *in vitro* from the *ncRAN* transcripts (Fig. 4). Though only the possible open reading frames (>150 bp) within the *ncRAN* cDNA were from n.t. 190 to 354 (55 amino acids) and from 293 to 469 (59 amino acids) in *Nbla10727*, none of the putative translation start sites contains the Kozak consensus sequence. In addition, these predicted protein products of 55 and 59 amino acids did not exhibit significant similarity to any other known protein or protein domain. Furthermore, *in vivo* transcription and translation of the full-length *ncRAN* did not lead to the synthesis of any peptide or protein (Fig. 4B), though endogenously and ectopically expressed *ncRAN* were easily detectable at mRNA level (Fig. 4A). Coincident with the above observation, the *ncRAN* protein product could not be detected using [35S]-methionine-labeling system *in vitro* (Fig. 4C).

Third, we performed sequence comparison of the *ncRAN* gene with genome sequences of other species and found it has high similarity (>90% identity in nucleotides) with primates including orangutan, chimpanzee and rhesus, but not those with mice and rat (Fig. 5). We also searched for the possible long open reading frames of *ncRAN* homologs in these highly similar species, resulting in failure. The highly conserved sequence similarity only with primates may
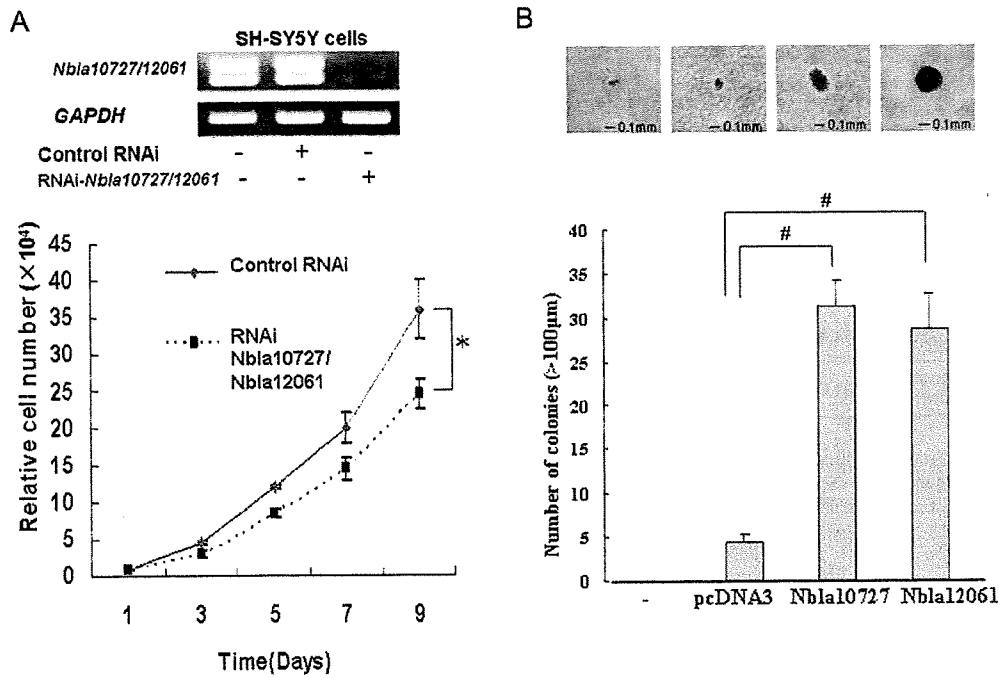
Figure 3. *ncRAN* is involved in inducing enhancement of cell growth in neuroblastoma cells and transformation of NIH3T3 cells. A, Knockdown of *ncRAN* suppress cell growth in SH-SY5Y neuroblastoma cells. SH-SY5Y cells were transfected with expression plasmid for siRNA against *ncRAN* termed pMuni-si*Nbla10727* or with the empty plasmid. On day 2, total RNA was prepared from the cells and subjected to RT-PCR. The expression of two splicing variants of *ncRAN* was knocked-down. At the same time, transfected cells were spread onto 24-well plates and the numbers of the cells at indicated time points were counted using hemocytometer and expressed as the mean ± SEM (n=3). *p<0.05. B, Overexpression of *ncRAN* promotes the malignant transformation of NIH3T3 cells. NIH3T3 cells transfected with pcDNA3, pcDNA3-*Nbla10727* and pcDNA3-*Nbla12061* were used to carry out the soft-agar assay as described in Materials and methods. Blank and mock-transfected NIH3T3 cells served as negative controls. $^#$p<0.01.
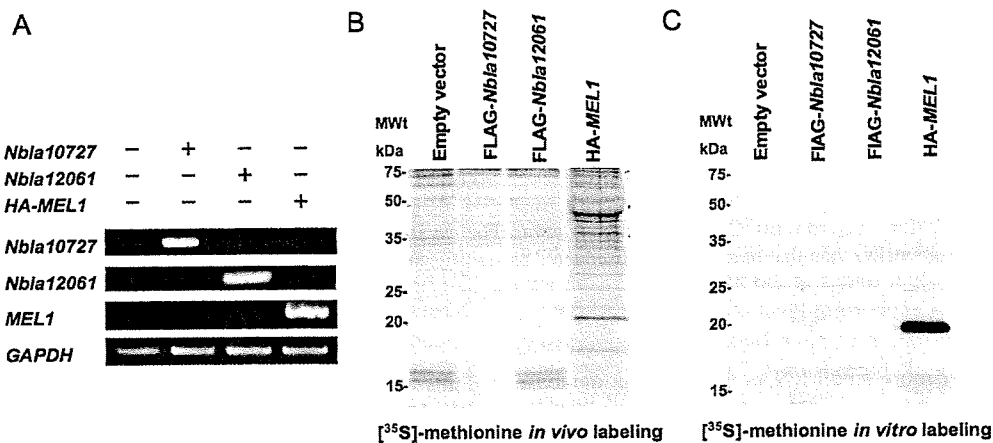

Figure 4. *ncRAN* is a non-protein-coding RNA. A, Ectopic expression of *ncRAN* transcripts in COS7 cells. The *ncRAN* expression vectors were transfected into COS7 cells and total RNA was subjected to RT-PCR. pcDNA3-HA-*MEL1* was used as a positive control. B, *In vivo* [^{35}S]-methionine labeling experiment. COS7 cells transfected with the indicated expression vectors were maintained in fresh growth media without methionine for 2 h and then cultured in the media containing [^{35}S]-methionine overnight. Cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody. Immune complex was washed extensively, resolved by SDS-PAGE and detected by autoradiography. Cell lysate prepared from COS7 cells transfected with pcDNA3-HA-*MEL1* were immunopricipitated with anti-HA antibody. C, *In vitro* translation assay. *In vitro* translation was performed in the presence of [^{35}S]-methionine according to the manufacturer's instructions. pcDNA3-HA-*MEL1* was used as a positive control.

suggest that *ncRAN* might be an evolutionarily developed non-coding RNA.

Finally, previous studies have shown that certain large non-coding RNAs are relevant to host RNAs that harbor small RNAs such as microRNA (miRNA) (18). Therefore, we made a search for sequences of known miRNAs in conserved regions within the *ncRAN* locus, but none were identified. These results inferred that the *ncRAN* transcript might not be
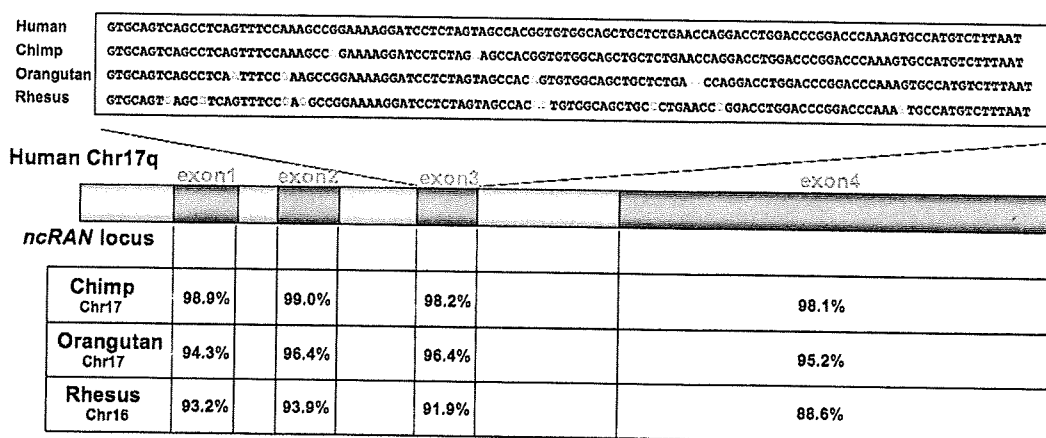
Figure 5. Schematic representation of *ncRAN* sequence conservation in primates. Sequence conservation in *ncRAN* gene locus among human and primates is indicated. Nucleotide sequences of exon3 of *ncRAN* in primates are indicated by numbers in brackets. Genomic sequences within the highly conserved sequence are marked black; mismatches are marked pink. % identities to humans are shown below for each exon. Other lower species, such as mouse, rat, dog, cow, horse, zebrafish, or *C. elegans*, do not have *ncRAN* in their genomes.

Table II. Multivariate analyses of *ncRAN/Nbla10727* mRNA expression as well as other prognostic factors in primary neuroblastomas.

Factor	n	p-value	q-value	H.R.	C.I.
Age (>12-month vs. <12-month)	45 vs. 25	0.0096		3.4	(1.2-9.9)
ncRAN expression	n=70	0.0015	0.0281	3.6	(1.7-7.9)
Age (>18-month vs. <18-month)	40 vs. 30	0.0150		2.9	(1.2-7.1)
ncRAN expression	n=70	0.0023	0.0361	3.5	(1.6-7.8)
Stage (1, 2, 4s vs. 3, 4)	42 vs. 28	<0.0001		8.0	(2.9-14)
ncRAN expression	n=70	0.0457	0.3151	2.4	(1.0-5.6)
Origin (adrenal vs. non-adrenal)	27 vs. 43	<0.0001		9.1	(2.6-33)
ncRAN expression	n=70	0.0107	0.1335	2.8	(1.3-6.1)
MYCN expression	n=70	0.0003		2.0	(1.4-2.8)
ncRAN expression	n=70	0.0035	0.0470	3.3	(1.5-7.3)

n, number of samples; H.R., hazard ratio; C.I., confidence interval. The q-value denotes estimated false discovery rate if all genes whose p-values are equal to or smaller than that of *ncRAN* are discovered as significant (17).

processed to one or more small RNAs. In addition, database search did not identify genes with anti-direction to *ncRAN*, excluding the possibility that *ncRAN* is an antisense gene for certain known genes. Collectively, these results strongly suggested that the *ncRAN* transcript functions as a novel large non-coding RNA.

Discussion

In the present study, we used the combination of array-CGH (5) and gene expression profiling by using an in-house neuroblastoma-proper cDNA microarray (10) to identify genes that strongly correlate with chromosome 17q gain in aggressive neuroblastoma. Our array CGH analysis demonstrated three major genomic groups of chromosomal aberrations such as silent (GGS), partial gains and/or losses (GGP), and whole gains and/or losses (GGW). Correlation analysis revealed that the global feature of the aberrations was maximally correlated with the gain of the long arm of chromosome 17 and with the gain of a whole chromosome 17, therefore the genomic groups GGP and GGW were defined by the status of aberration, by 17q gain and 17 whole chromosomal gain occurred in chromosome 17, respectively (5). Survival analysis for each genetic group suggested that 17q gain was a characteristic and prognosis-related event in primary neuroblastomas. Therefore, we searched for genes that were expressed significantly higher in primary neuroblastomas of GGP compared to that of GGS and GGW and finally found a novel gene *ncRAN* mapped on 17q25.1. The level of its mRNA expression was strongly correlated with the status of chromosome 17 (Table I and Fig. 1B) as well as with patient survival (Table II and Fig. 2).

To our surprise, our results suggested that *ncRAN* is a large non-coding RNA. Non-coding RNA is a general term for functional and untranslatable RNAs. Increasing evidence has shown that they play important roles in a variety of biological events such as transcriptional and translational gene regulation, RNA processing and protein transport (18,19). Recently, the numerous miRNAs, a class of small non-coding RNAs, have been identified, and miRNA-expression profiling of the human tumors has identified signatures in relation to diagnosis, staging, progression, prognosis and response to treatment (19). On the other hand, another class of non-coding RNAs named as the large non-coding RNA, which are usually produced by RNA polymerase II and lack significant and utilized open reading frame, receives relatively little attention. However, recently, increasing number of studies have provided evidence that large non-coding RNAs also play important roles in certain biological processes of the cancers, such as acquisition of drug resistance, transformation, promoting metastasis and inhibition of tumor development (19). In addition, certain candidate non-coding RNAs were isolated from the tissue- and stage-specific libraries, suggesting a possible involvement of non-coding RNAs in development and tumor cell differentiation (20). Given that *ncRAN* was identified from the cDNA libraries generated from different subsets of primary neuroblastomas, it is possible that *ncRAN* might be involved in carcinogenic processes as well as development and differentiation of normal neurons.

In conclusion, we identified a novel large non-coding RNA transcript, *ncRAN*, mapped to the region of 17q gain frequently observed in aggressive neuroblastomas. The levels of *ncRAN* expression are relatively low in normal nerve tissues including adrenal gland, whereas they are upregulated in advanced neuroblastomas with gain of chromosome 17q. From our functional analyses, *ncRAN* appears to act like an oncogene. Notably, knockdown of *ncRAN* with siRNA was able to significantly repress the cell growth in SH-SY5Y neuroblastoma cells with 17q gain as well as high endogenous level of *ncRAN*. Considering emerging evidence on the large non-coding RNAs regulating transcription of other genes (19), the present results not only contribute to further understanding of the molecular and biological mechanism of neuroblastoma genesis, but also provide a potential target for new diagnostic and therapeutic intervention in the future.

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ORIGINAL ARTICLE

Distinct role of ShcC docking protein in the differentiation of neuroblastoma

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The biological and clinical heterogeneity of neuroblastoma is closely associated with signaling pathways that control cellular characteristics such as proliferation, survival and differentiation. The Shc family of docking proteins is important in these pathways by mediating cellular signaling. In this study, we analysed the expression levels of ShcA and ShcC proteins in 46 neuroblastoma samples and showed that a significantly higher level of ShcC protein is observed in neuroblastomas with poor prognostic factors such as advanced stage and MYCN amplification (P<0.005), whereas the expression level of ShcA showed no significant association with these factors. Using TNB1 cells that express a high level of ShcC protein, it was demonstrated that knockdown of ShcC by RNAi caused elevation in the phosphorylation of ShcA, which resulted in sustained extracellular signal-regulated kinase activation and neurite outgrowth. The neurites induced by ShcC knockdown expressed several markers of neuronal differentiation suggesting that the expression of ShcC potentially has a function in inhibiting the differentiation of neuroblastoma cells. In addition, marked suppression of in vivo tumorigenicity of TNB1 cells in nude mice was observed by stable knockdown of ShcC protein. These findings indicate that ShcC is a therapeutic target that might induce differentiation in the aggressive type of neuroblastomas.
Oncogene (2009) 28, 662–673; doi:10.1038/onc.2008.413; published online 10 November 2008

Keywords: Shc family; ERK; neuroblastoma; differentiation; RNAi

Introduction

Neuroblastoma is the most common pediatric solid tumor derived from the sympathoadrenal lineage of neural crest and its clinical and biological features are heterogeneous. Some types of neuroblastomas show favorable outcomes with spontaneous differentiation or regression by minimum treatment, whereas other types have malignant characteristics with metastasis and

resistance to chemotherapy. Age of onset, tumor volume, presence of metastasis, pathological features and amplification of the N-myc gene are important prognostic factors of neuroblastoma. Previously, it was reported that the differential expression of Trk family receptors might contribute to clinical and biological outcomes of neuroblastomas (Nakagawara et al., 1993; Nakagawara and Brodeur, 1997) whereas the cellular signaling involved in the regulation of the aggressiveness of neuroblastoma is largely unknown.

The Shc family of docking proteins is important in signaling pathways mediating the activation of various receptor tyrosine kinases (RTKs) such as the Trk family triggered by extracellular stimulations, to specific downstream molecules. The Ras–extracellular signal-regulated kinase (ERK) pathway and the phosphoinositide-3 kinase (PI3K)–Akt pathway are the most common signals regulated by Shc family proteins, representing important functions in cellular proliferation, survival and differentiation.

The Shc family has three members, ShcA/Shc, ShcB/Sli/Sck and ShcC/Rai/N-Shc encoded by different genes (Nakamura et al., 1996; O'Bryan et al., 1996; Pelicci et al., 1996). ShcA protein having three protein isoforms, p46, p52 and p66, is ubiquitously expressed in most organs except the adult neural systems, whereas ShcC (p52 and p67 isoforms) are exclusively expressed in the neuronal system (Sakai et al., 2000). In the central nervous system, ShcA expression is most significant during embryonic development with sudden decrease after birth. On the other hand, ShcC expression is remarkably induced around birth and maintained in the mature brain. The Shc family molecules have a unique PTB–CH1—SH2 modular organization with two phosphotyrosine-binding modules, PTB and SH2 domains, which recognize various phosphotyrosine-containing peptides with different specificities. CH1 domains contain several tyrosine phosphorylation sites that recruit other adaptor molecules such as Grb2. Functional analysis of ShcB and ShcC on the neuronal signal pathway indicate that these proteins in neuronal cells potentially regulate epidermal growth factor (EGF) or nerve growth factor (NGF) signaling in a similar fashion to ShcA (O'Bryan et al., 1996; Nakamura et al., 1998).

Major parts of neuroblastoma cell lines show the expression and tyrosine phosphorylation of ShcC protein, but its effect on the biology of tumor cells

remains to be elucidated. We have recently shown that constitutive tyrosine phosphorylation of ShcC is induced in a subset of neuroblastoma cells by the activation of anaplastic lymphoma kinase (ALK) owing to *ALK* gene amplification and the constitutively activated ALK–ShcC signal pathway could induce cell survival, anchorage-independent growth of the cells and progression of tumors (Miyake *et al.*, 2002, 2005). In our study, significant amplification of *ALK* was observed in 3 of 13 neuroblastoma cell lines and in only 1 of 85 cases of human neuroblastoma samples (Osajima-Hakomori *et al.*, 2005). Considering these results, it was suspected ShcC might also contribute to the signal pathway associated with the tumor behavior in ALK-independent manners in majority of neuroblastoma cells.

In a recent report, high expression of ShcC mRNA was shown to be a poor prognostic factor in neuroblastoma patients through the semiquantitative reverse transcriptase -PCR analysis of tissue samples (Terui *et al.*, 2005), suggesting the possibility that ShcC protein might be causative of tumor progression in neuroblastoma patients. In the current study, we examined the expression levels of ShcC protein in tumor samples of 46 neuroblastoma patients and confirmed the significant association of the

expression levels of ShcC protein with several factors linked to unfavorable outcome of neuroblastoma. Furthermore, we investigated the functions of ShcC in cell proliferation, differentiation and *in vivo* tumorigenicity of neuroblastoma cells by knockdown of ShcC expression in neuroblastoma cell lines expressing a high level of ShcC without *ALK* amplification.

Results

Expression and tyrosine phosphorylation of ShcC in neuroblastoma cell lines

At first, the expression of ShcA and ShcC was analysed in 11 neuroblastoma cell lines using each specific antibody (Supplementary Figure A) along with DLD-1 as a control, which is known to express ShcA protein (mainly p46ShcA and p52ShcA), but not ShcC protein (Figure 1a). The three cell lines with *ALK* gene amplification (NB-39-v, Nagai and NB-1: Group A) expressed ShcC at a moderate level in contrast to their significant phosphorylation so that ALK–ShcC complex is mediating the dominant oncogenic signal (Miyake *et al.*, 2002; Osajima-Hakomori *et al.*, 2005). Other
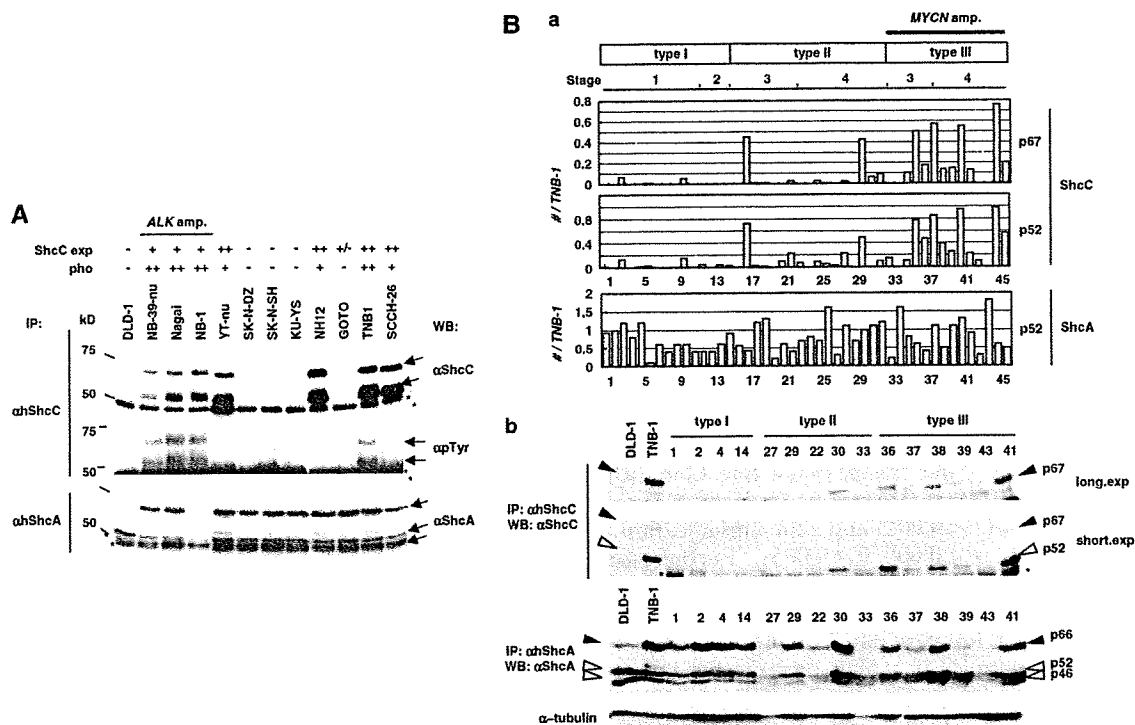

Figure 1 (A) Expression and tyrosine phosphorylation of ShcC in neuroblastoma cell lines detected by specific antibody. The expression of ShcA (lower panel), ShcC (upper panel) and tyrosine phosphorylation of ShcC was analysed in 11 neuroblastoma cell lines including the cell lines with anaplastic lymphoma kinase (*ALK*) gene amplification (*ALK* amp.) along with DLD-1 as a control. Lysates were immunoprecipitated and then immunoblotted with antibodies against the indicated molecules. The levels of expression/ phosphorylation of ShcC are indicated above. Asterisks show heavy chains of immunoglobulin. Positions of molecular mass markers (kDa) are shown to the left. (B) Expression of ShcC and ShcA in the tissue samples of three subsets of neuroblastoma patients. (a) Expression levels of ShcC/ShcA in the samples of 46 neuroblastoma patients were detected by western blotting being compared to the level of expression in TNB-1 cells (= 1.0) as an internal control among each experiment and was corrected by each expression level of a-tubulin. (b) Expression of ShcC (upper panel)/ShcA (middle panel) of representative samples of each subset were detected on a filter. The exposure time of the filter onto X-ray films was different to detect between p52ShcC (short exposure: lower panel) and p67ShcC (long exposure: upper panel). Each isoform of ShcC/ShcA is indicated by opened or filled triangles. Asterisks show heavy chains of immunoglobulin.

neuroblastoma cells with a single copy of the *ALK* gene were divided into two groups, one with considerably high levels of ShcC expression (YT-v, NH-12, TNB-1 and SCCH-26: Group B) and the other with almost no ShcC expression (KU-YS, SK-N-DZ, SK-N-SH and GOTO: Group C). Most of the cells in the Group B showed a morphological tendency to aggregate each other and rather low adhesion to the culture plate, compared with the cells of the Group C (data not shown). The degrees of ShcC phosphorylation in the cells in Group B appeared to be lower than the cells with *ALK* amplification (Figure 1A, middle panel). In contrast to ShcC, the expression of ShcA was within similar levels among neuroblastoma cell lines (Figure 1A, lower panel).

The expression level of ShcC is prominent in tissue samples of poor risk neuroblastoma patients
Next we analysed the expression of ShcA and ShcC protein in 46 primary human neuroblastoma specimens using each specific antibody. These tissue samples were classified into three subsets using Brodeur's classification; type I (stage 1, 2 or 4S; a single copy of *MYCN*), type II (stage 3 or 4; a single copy of *MYCN*) and type III (all stages; amplification of *MYCN*) (Brodeur and Nakagawara, 1992; Ohira *et al*., 2003). The expression level of ShcA and ShcC in western blotting was standardized by intensity of α-tubulin within each filter, standardized by the amounts in TNB-1 cells as an internal control among different filters and statistically evaluated from at least two independent western blots for each sample (Table 1). We found that there is a significant difference in the expression levels of ShcC among the subsets of neuroblastomas. In the group of type II and type III, the expression level of ShcC protein was substantially higher than that in the type I group (Figure 1Ba). Both isoforms of ShcC, p52ShcC and p67ShcC, showed similar patterns of expression. As shown in Table 2, the expression level of ShcC has a significant correlation with several clinical factors including late onset of the disease (later than 12 months) (p52/p67: $P < 0.001/P = 0.015$), advanced clinical stage (stages III and IV) ($P < 0.001$) and gene amplification of *MYCN* (p52/p67: $P < 0.002/P = 0.005$). Furthermore, most of the samples from the patients who died within 12 months after the onset of the disease showed significantly higher levels of ShcC expression than the other group of samples in which patients lived longer than 12 months (p52/p67: $P = 0.006/P = 0.009$). In contrast, variable expression levels of both isoforms of ShcA protein, p52 and p66, were observed in neuroblastoma samples with no significant difference among three subsets of clinical group ($P > 0.05$) (Table 2; Figure 1Ba). The results of representative samples from each subset are shown in Figure 1Bb. These data indicate that the expression of ShcC protein is significantly associated with multiple prognostic factors of neuroblastoma, suggesting that ShcC has specific functions in malignant phenotypes of neuroblastoma presumably by modulating cellular signaling.

Biological effects of ShcC downregulation on TNB-1 cells
To elucidate the biological functions of ShcC in the tumor characteristics causing unfavorable outcomes of neuroblastoma patients, we investigated the effects of ShcC knockdown on the cellular biology and signal transduction in one of the neuroblastoma cell lines, TNB-1, which expresses a high level of ShcC protein with no *ALK* amplification. The expression of ShcC and ShcA was suppressed by RNA interference using two independent sets of specific small interfering RNA (siRNA) oligonucleotides corresponding to ShcC and ShcA, respectively (Figure 2Aa). The growth rate of TNB-1 cells transfected with the ShcA siRNA was severely suppressed (Figure 2Ab), owing to impaired ability of proliferation and survival, which is consistent with previous reports (Ravichandran, 2001). ShcC-knockdown cells showed a relatively weak effect on growth rate in the normal culture condition (Figure 2Ab).

Downregulation of ShcC induces neurite outgrowth and increases differentiation-related markers in TNB-1 cells
ShcC knockdown caused morphological changes to rather flat and spindle shape and neurite extension within 24 h after transfection of ShcC siRNA (Figure 2Ba). These neurite-bearing cells express higher amount of microtubule-associated protein 2 (MAP-2), growth-associated protein 43 (GAP-43), a protein expressed in the growing neurites, and chromogranin A (Chr-A; Figure 2Bb), markers of neuronal differentiation (Giudici *et al*., 1992) than the control cells. On the other hand, TNB-1 cells treated with ShcA siRNAs showed no remarkable change compared with the control cells, relatively round with small processes attached to the dish surface (Figure 2Ba). These results suggest that the endogenous ShcC negatively affects neurite outgrowth and differentiation of TNB-1 cells.

Persistent activation of ERK1/2 due to ShcC downregulation induces neurite outgrowth in TNB-1 cells
Neuronal differentiation is closely associated with mitogen-activated protein kinase (MAPK)/ERK kinase (MEK)/ERK and PI3K–AKT pathways and both of them might be controlled downstream of Shc family signaling. Downregulation of ShcA induced the suppression of extracellular signal-related kinase 1/2 (ERK1/2) and AKT pathways at 48 h after transfection of siRNA, nevertheless ShcC downregulation apparently elevated the base level of ERK phosphorylation and slightly enhanced AKT activation (Figure 3a). This elevation of ERK phosphorylation sustained until 96 h after transfection of siRNA. Similar effect on the ERK activation was also observed in NH-12 and YT-v cells, which express high amount of ShcC (Supplementary Figure B). It is reported that sustained activation of ERK is responsible for neurite outgrowth and differentiation of PC12 cells (Qui and Green, 1992). To investigate whether neuronal extension of TNB-1 cells by ShcC RNAi was induced by sustained activation of ERK, the effect of MEK inhibitor, PD98059, on the