する神経芽腫は32症例であった．このうち29例は無病生存例であり，症例の臨床転帰良好に対するこの群の特異性は91%であり，「低リスク神経芽腫」として層別が可能であった．

3）「診断時の腫瘍進展度」も臨床転帰に関与する重要な指標（図1）

「INPC で FH」および「H/t 高発現」で「診断時 I, II 期」の限局腫瘍症例の PFS は 100%であった（Low risk 群）．一方，「INPC で UH」あるいは「H/t 低発現」を有する「High risk 群」に分類された症例の PFS は 39%（32/83）であった．しかしこれら「High risk 群」でも I, II 期の限局腫瘍症例の PFS は 91%（10/11）と良好で，この群の MYCN 増幅の一例にも再発を認めていない．これに対し同じ「High risk 群」で III, IV 期腫瘍症例の PFS は 28%（19/67）であった．

臨床予後良好が期待できる「INPC で FH」および「H/t 高発現 / 中間発現」腫瘍でも III, IV 期の腫瘍症例の PFS は 67%(31/46)であり Low risk 群と High risk 群の中間の PFS であったため，これらを「Intermediate risk 群」とリスク評価を分類した．

最も予後不良な群は「INPC で UH」で「H/t 低発現」の悪性性状の二指標を持ち病期 III, IV 期症例の PFS は 17%(4/24)であった．

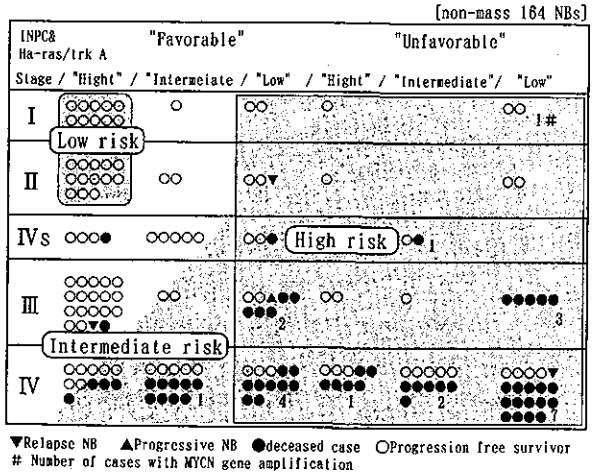

図1　INPC 組織所見および Ha-ras/trkA 遺伝子発現，MYCN 遺伝子増幅，腫瘍病期による神経芽腫性状の層別化

IV 考　察

1980 年代から分子生物学的手法により神経芽腫の多様性が次第に明らかになり，Brodeur 等により生物学的指標に基づき 3 つのサブタイプの神経芽腫の概念が提起された[9]．しかし，臨床的にどの程度の特異性，感度でそれぞれの指標が臨床的に機能するかを検討した報告はない．代表的予後不良指標である「MYCN 増幅」の高い特異性は広く知られているが，臨床転帰不良例全体に対する感度は半数にとどまり，「MYCN 増幅」は予後不良を強く示唆する一方向の指標であり，今回の検討でも示されたように「非増幅」は必ずしも予後良好を示すものでなかった．これに対し「INPC の UF」，「H/t 低発現」は「MYCN 増幅」と同様症例の臨床転帰不良と関連するが，「INPC が FH」と「H/t 高発現」は共に予後良好指標として有意であり，両者は予後良／不良の双方向性を有する独立指標であった．これまでの臨床的な検討結果からも神経芽腫性状を規定する因子は複数存在すると考えられている．これまでに遺伝子レベルの指標として細胞増殖に関与する「MYCN 遺伝子」，神経細胞の生存／アポトーシスに関与する「trk A 遺伝子」[10]，細胞分化／非アポトーシス機構で細胞死に関与する「Ha-ras 遺伝子」[11]などが報告されている．

今回の検討で予後不良指標である「MYCN 増幅」，「INPC が UH」，「H/t 低発現」のいずれかを有する神経芽腫は全臨床転帰不良症例の 83% を占め「High risk 神経芽腫」と規定する事ができた．このように三指標を検討することにより予後不良症例に対する感度が向上した．一方，今回の検討で「MYCN 非増幅」「INPC が FH」「H/t 高発現」の 3 条件を全て有する神経芽腫の無病生存に対する特異性は 91% であり「Low risk 神経芽腫」と規定する事もできた．これら指標の組み合わせによる神経芽腫性状の評価は臨床的に極めて有用である事が示された．

図 1 に提示された 164 例の神経芽腫のうち 104 症例で「MYCN 遺伝子」が検討され 22 腫瘍にその増幅が認められた．この 22 症例中 21 症例

(95%) は「INPC が UH」および「H/t 低発現」で規定される High risk 神経芽腫群に含まれ，三者はお互い予後予測に補完的に機能する事が示された．

更に興味ある結果として図 1 に示された High risk 神経芽腫症例でも，病期 I および II 期の 11 例中 10 例の臨床転帰は良好であった．リスクを有する腫瘍でも「診断時の腫瘍病期」により更にその臨床予後が層別され，早期治療介入の効果を窺わせる結果であった．診断時の腫瘍進展度は外科的処置をはじめ治療介入の難易度を示す重要な予後関連因子の一つであると考えられた．

その一方で，「INPC の FH」で「H/t 遺伝子の一方が低発現で他方が高発現（中間発現群）」の腫瘍症例の PFS は 63% (15/24) でこの死亡例の中の 9 例は IV 期の進展腫瘍群に集中していた．更に「INPC が FH」「H/t 高発現」腫瘍でも III 期および IV 期の腫瘍症例では PFS が 80% (24/30) で I 期 II 期の限局腫瘍群（100% 生存の Low risk 群）に比べ臨床転帰に差が見られた．一般に乳児期 IVs 期腫瘍は良好な臨床経過と考えられているが，今回の検討でも IVs 期腫瘍症例の PFS は 79% (11/14) で 1 例の MYCN 増幅例が含まれていた．これまで我々が行ってきた非マス神経芽腫の集計結果[5]では IVs 期腫瘍症例の臨床転帰は III 期症例と同じであり進展腫瘍の一つとして予後関連因子による性状評価を行い治療方針を決めるべきと考えている．以上の結果より「INPC の FH」「H/t 高発現／中間発現」で病期 III, IVs, IV 期の神経芽腫を「Intermediate risk」腫瘍群と規定した（図 1）．「Intermediate risk」神経芽腫は現行の治療に比較的反応良好な腫瘍群と我々は考えている．腫瘍進展度を考慮すれば治療介入の効率のよさから「INPC 所見の UH」「H/t 低発現」腫瘍でも I 期 II 期の限局腫瘍の場合は適確に治療された後の進展リスクは低くなるものと考えられた．以上の所見よりこの群のリスク評価は今後の症例集積により緩和変更される可能性がある．「risk」区分の境界設定には更なる検討を要するがこれまでの結果から現状での神経芽腫のリスク区分として図 1 を提示した．

V まとめ

「MYCN 増幅の有無」「INPC の FH／UH 所見」「H/t 高 / 低発現」「診断時の腫瘍進展病期」など複数の指標により高い特異性と感度を有する神経芽腫性状の評価の臨床的有用性が示された．

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症　例

胆道閉鎖症に肝細胞癌を合併し生体肝移植術後5年後に後腹膜リンパ節に再発を認めた1例

Recurrence of hepatocellular carcinoma 5 years after liver transplantation in a child with biliary atresia and hepatocellular carcinoma

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要　旨

患児は胆道閉鎖症術後胆汁排泄あるも黄疸は持続していた．7才4カ月時に発熱，腹部膨満を主訴に入院となった．T-bil 7.1mg/dl,D-bil 5.6mg/dl,AFPは1958ng/mlと異常高値でCTで肝右葉内に被膜を有する3つの腫瘤像を認め，生検にて中分化型肝細胞癌と診断された．陽子線照射により肝細胞癌を十分にコントロールした後に生体部分肝移植手術を施行した．移植肝および肝癌の経過は良好であったが移植後5年を経過したところで，はじめてAFPの再上昇を認めた．CTにて移植肝内には腫瘤像は認めなかったが，腹腔動脈周囲のリンパ節転移を認めこれを切除した．しかし，その後移植肝に多発性の肝内転移が発生し肝移植後6年1ヶ月で失った．

Key words：**胆道閉鎖症，肝細胞癌，肝移植，再発**
liver transplantation, biliary atresia, hepatocellular carcinoma,recurrence

はじめに

胆道閉鎖症術後に肝細胞癌が合併した症例に対し生体肝移植手術を施行した．移植後拒絶反応もなく5年経過した後に肝細胞癌の再発が認められた．肝細胞癌は胆道閉鎖症術後の長期生存例が増加しつつある現在注意すべき合併症の1つである．5年後にはじめて転移が発見されたこの症例では移植時期を考える上で貴重な症例であると思われるので報告する．

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症　例

生体肝移植までの経過：

在胎43週3,530gにて出生．84日齢に胆道閉鎖症の診断で肝門部腸吻合を施行した．6か月時に癒着性イレウスにて剥離術施行．その後胆汁排泄あるも黄疸持続したまま経過観察されていた．6才で食道静脈瘤に対し内視鏡的硬化療法を施行した．7才で腹水貯留が始まり発熱・黄疸・腹部

図1　CT(7才入院時)
S5,S 7に被膜を有する最大径39mmの低吸収性腫瘍像を認めた.

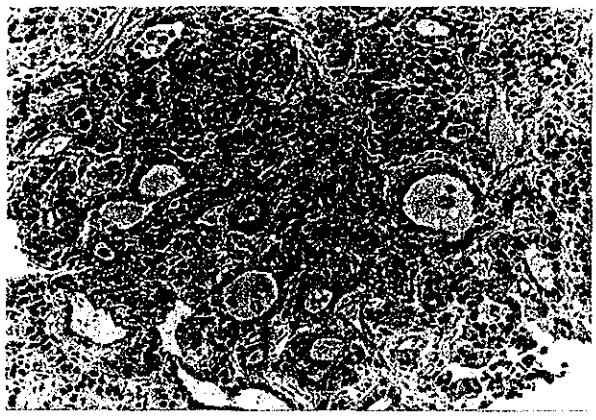

図2　肝針生検(7才7ヵ月) HE, × 50
偽腺管構造を有する中分化型肝細胞癌

膨満のため入院となった.

家族歴・既往歴：特記すべきことなし

現　症：身長114cm, 体重20.3kg, 肝（右季肋下4.5cm・弾性硬), 脾（左季肋下5cm)

入院時検査所見：

血液一般検査　WBC 2100/mm³, Hb 8.0g/dl, Plt 8 × 10⁴/mm³

肝炎ウイルス HBsAg(-), HBsAb(-), HCVAb(-)

血液生化学検査 TP 5.0g/dl, Alb 2.3g/dl,T-bil 7.1mg/dl, D-bil 5.6mg/dl,AST 159 U/l, ALT 61 U/l, AFP 1958ng/ml, ICG R15(%) 27%

入院後経過：

CTにて肝内S5, S7に被膜を有する3つの腫瘤像(10 × 10mm, 19 × 19mm, 39 × 36mm)を認めた（図1). エコーガイド下に肝生検を施行し中分化型肝細胞癌と診断された(図2). 1カ月間でAFPは4294ng/mlまで上昇し他臓器には転移がないため, 肝内腫瘍をコントロールするため右葉の腫瘍の部に陽子線照射(3.5Gy × 16回)を行いAFPは296ng/mlまで下降した. 画像診断上も腫瘍の縮小傾向を認め他臓器への転移はなかった.

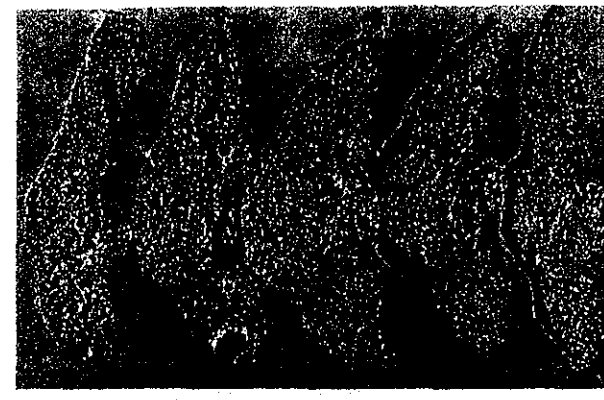

図3　摘出肝(8才1ヵ月)
S7　24 × 28mm　単結節型
被膜形成あり　門脈・肝静脈浸潤なし
腫瘍組織内には陽子線照射後の壊死組織が目立つが腫瘍細胞は残存した.
S2　15 × 15mm　単結節型
被膜形成あり　門脈・肝静脈浸潤なし

図4　CT(13才2ヵ月)
腹腔動脈周囲にエンハンスされる転移リンパ節（矢印）を認める. 移植肝内には腫瘤像は全く認められなかった

生体肝移植と術後経過：

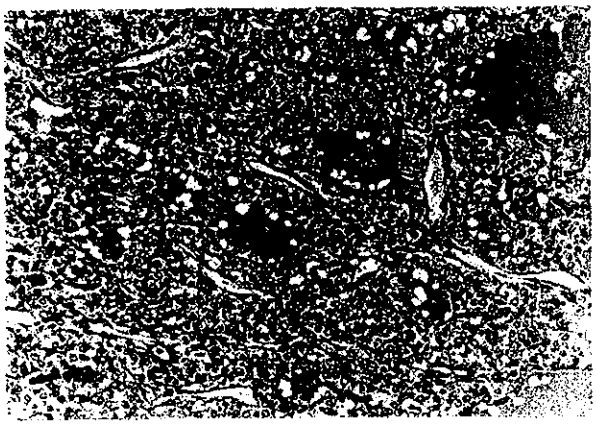

図5　転移リンパ節(13才2ヵ月)HE, × 50
やはり偽腺管構造を有する中～低分化型肝細胞癌であった.

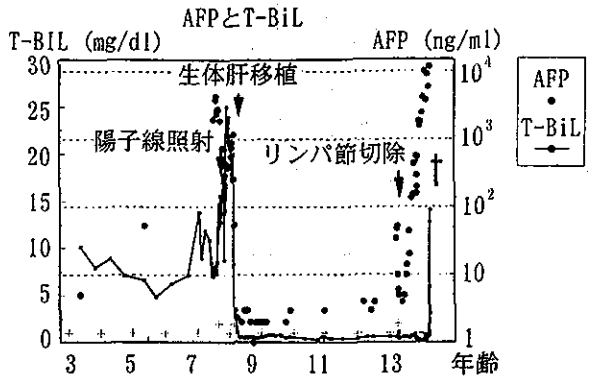

図6　経過表
5年間にわたりAFPは10ng/ml以下で総ビリルビン値も正常であった.

8才1カ月時に母をドナーとした生体部分肝移植手術を施行. 母の左葉を同所性に移植した. 移植時アドリアマイシン8mgの静注を行った. 術中腹水の迅速細胞診では腫瘍細胞は認められなかった. 肝移植直前のAFPは1171ng/mlであった. 摘出肝ではS7に直径2.5cm(中分化型), S2に直径1.5cm(低分化型)の腫瘍を認めた(図3)肝内門脈・肝静脈への浸潤は認められなかった. 術後20日でAFP値は10ng/ml以下となり, 画像上も再発は認めず肝機能も良好であった. 免疫抑制剤はサイクロスポリン(途中からネオーラル)血中濃度は50－100ng/ml, 極少量のソルメドロール(移植後4年で中止)を使用, また移植後けいれん発作予防のためテグレトール0.4g/日を内服して外来で経過観察されていた.

13才2カ月にAFP 34ng/mlとなり, CTにて精査したところ腹腔動脈周囲のリンパ節転移を認めた(図4). 移植肝には転移・再発を疑われる所見はなかった. 腹腔動脈周囲のリンパ節切除術(大きさ40×33×25mm, 重さ16g)を施行した(図5). その後AFP値は10ng/ml以下となり経過観察されていた. 4ヶ月後の13才6カ月時よりAFP値が再び上昇傾向となった. 脳・肺・骨・リンパ節には転移は認めないが, CTでは肝内(移植肝左葉内側区を中心)に多発腫瘤像を認めた. その後さらに腫瘍の増殖が続き移植肝は殆ど腫瘍に置き換わり14才3カ月で腫瘍死した(図6). 剖検は得られなかったが画像上は腫瘍は腹腔内, 肺, 骨, 脳など肝外には確認できなかった.

考　案

近年我が国においても胆道閉鎖症術後の肝細胞癌の合併例の報告例が見られるようになった(表1)[1)-12)]. 胆道閉鎖症術後の長期生存例が増加しつつある現在注意すべき合併症の1つである. HCV感染を経て肝細胞癌を合併した報告例もある[2)]. 胆道閉鎖症に続発する小児肝癌例の腫瘍細胞の形態に特別な特徴は知られていない[13)]. HCCと肝芽種の鑑別はHCCには肝硬変が合併すること, 腫瘍細胞が正常肝細胞より大きいこと, 多層の細胞索, 核分裂像がよく見られることなどである[14)]. 胆道閉鎖に発生する肝癌については早期診断に心がける必要がある. 肝硬変患者にならって胆道閉鎖症術後の患児にも一般肝機能検査に加えて, 腫瘍マーカー, 画像診断による定期的検査を実施する. HCCに特異的な腫瘍マーカーはα-feto-protein(AFP)である. 低値ながら漸増する場合はHCCの発生を疑う. AFP値が異常値の場合はレクチン分画(L3)でHCC特異性を調べる必要がある. 定期的な超音波(US)を年2回程度は行い, 経過観察中にAFP値の上昇や低エコーか高エコー性の類円形結節が出現すればHCCを疑いCTやMRI検査を行う必要がある.

肝細胞癌に対する肝移植の適応については,

Mazzaferroの基準[15]（単発なら最大径5cmまで，3個以内で最大径3cm以下）が参考になる．これはMilan reportとも呼ばれている．我が国での脳死肝移植の適応基準は極めて厳しく細小肝癌（径2cm以下1個）であった．田中らは肝癌に対する肝移植適応条件を以下の4点で決定している[16]．(1)ほかに治癒を期待できる治療方法がない，(2)肝外病変がない，(3)肝静脈や門脈主要枝への浸潤や腫瘍塞栓がない，(4)肝内腫瘍結節の数や径による適応条件は設定しない，である．本症例はこの基準に合致しているが，当初は肝癌が多発して大きさも大きいことから肝移植の適応はないとされた．そこで陽子線照射を行って腫瘍の縮小をはかりAFP値の明らかな低下がみられたので肝移植にふみ切った．手術の工夫はまずレシピエントを開腹して局所リンパ節や横隔膜浸潤の有無などの所見を確認し，転移病巣がみられない場合にドナーに麻酔を導入して手術を行うこととしている．通常の全肝摘除と異なる点として，肝血流を遮断して細胞の散布を予防することを目的に，開腹して早期に肝門部剥離を行い，門脈の断端と下大静脈の端側吻合による門脈下大静脈シャントを作製しておくことを原則としている[16]．

我々の症例は陽子線照射後肝移植手術を施行し治癒が期待されたが，術後5年後に再発した．胆道閉鎖の移植例ではこれまでに肺転移で術後10週で死亡した症例があるが5年経過後の再発例はなかった[5]．肝細胞癌に対し肝移植を行った小児例の報告では，19例のうち5年生存率は63%でTNM分類のStage Ⅳ以上また門脈浸潤例の予後は非常に悪い．うち6例（32%）は肺，脳，移植肝に再発したため死亡した[17]．再発の原因はもともとの肝細胞癌がどこかに残っていたものが出てきた可能性が高い．再発部位としては肺・肝・脳・骨が一般的である．この他可能性は少ないが術後発作予防のため使用していた抗けいれん剤には添付文書によると肝腫瘍発症の危険性が動物実験であるとされており注意を要する．初回腫瘍診断確定のための針生検は腫瘍播種，腹壁再発の危険性が指摘されているが本症例では腹膜播種や腹壁再発は認められていない．ドナー由来の癌発生が移植例にて報告されているが，大動脈周囲のリンパ節に再発した本症例では全く考えられない．肝移植術前後の抗ガン剤投与に関してはこれまで

表1　胆道閉鎖症と肝細胞癌の合併例　文献1）～12）

	報告者	報告年	性	BA手術	発症年例	AFP値	TBil	肝癌の治療	予後
1	Okuyama	1965	M	——		(ng/ml)	(mg/dl)	——	死
2	Abolson	1965	F	——				——	死
3	Fish	1966	M	——	9月			切除	死
4	Deoras	1968	F	——	6才		19	——	死
5	Van Wyks	1972	F	あり	12才	高値	3.5	肝移植	死(再発)10週
6	Van Wyks	1972	F	あり	4才	高値		肝移植	生18ヶ月
7	田村	1993	F	あり	16才	50000↑	7.7	5'FUDR静注	死
8	Kawarasaki	1994	F	あり	12才	0-5	12	肝移植	生43ヶ月
9	Kohno	1995	F	あり	5才	2850	15-20	腫瘍内エタノール注	死
10	Esquivel	1994		あり	11才	?	(incidental)	肝移植	生44ヶ月
11	Superina	1996		あり	12才		黄疸なし	肝移植	生36ヶ月
12	Tatekawa	2001	M	あり	8才	176442		piraubicin→肝移植 doxorubicin,uracil-futraful	生60ヶ月
13	Tatekawa	2001	F	あり	10才	3.0>		肝移植 uracil-futraful	生33ヶ月
14	小豆畑	2003	M	あり	20才	215.3	1.02	肝動脈塞栓術（TAE）	死6ヶ月
15	自験例		M	あり	7才	1958	7.1	陽子線照射→肝移植	死(再発)73ヶ月

のところ一定の見解はなく治療に関しても今後さらに検討を要する.

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FENRETINIDE INDUCES SUSTAINED-ACTIVATION OF JNK/p38 MAPK AND APOPTOSIS IN A REACTIVE OXYGEN SPECIES-DEPENDENT MANNER IN NEUROBLASTOMA CELLS

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Fenretinide, which mediates apoptosis in neuroblastoma cells, is being considered as a novel therapeutic for neuroblastoma. The cytotoxic mechanisms of fenretinide, however, have not been fully elucidated. Sustained-activation of JNK and p38 MAPK signaling has been shown recently to have a pivotal role in stress-induced apoptosis. Whether fenretinide activates the signaling in neuroblastoma cells is not known. In the present study, fenretinide induced sustained-activation of both JNK and p38 MAPK in neuroblastoma cells. Pretreatment with the antioxidant L-ascorbic acid almost completely inhibited the accumulation of fenretinide-induced intracellular reactive oxygen species (ROS), activation of JNK and p38 MAPK and apoptosis. Intracellular ROS production and activation of stress signaling was not altered by fenretinide in resistant neuroblastoma cells. Our study demonstrates that in neuroblastoma cells, fenretinide induces sustained-activation of JNK and p38 MAPK in an ROS-dependent manner and indicates that JNK and p38 MAPK signaling might mediate fenretinide-induced apoptosis. Our results also indicate that suppression of the fenretinide-induced ROS productive system and the downstream JNK and p38 MAPK signaling pathways causes neuroblastoma cells to become resistant to fenretinide.

Key words: *fenretinide; neuroblastoma; apoptosis; reactive oxygen species; JNK; p38 MAPK*

Neuroblastoma (NB) is one of the common malignant solid tumors in childhood, arising from neural crest progenitors. Despite progress with multimodal therapies consisting of multidrug chemotherapy, surgical and radiation therapy, the prognosis of advanced NB remains poor.[1] Therefore, new therapeutic approaches are needed. Retinoic acids (RA), which are vitamin A analogs, have been shown to induce the differentiation of NB cells into mature neuronal cells.[2] It has been reported recently that oral administration of 13-cis RA after consolidated chemotherapy with stem cell transplantation improved the 3-year event-free survival of advanced NB patients.[3] Retinoic acids are expected to be used as new therapeutic agents against NB, in view of their relatively low toxicity.

N-(4-hydroxyphenyl) retinamide, also called fenretinide (FR), has cytotoxic activity against various tumor cells including NB.[4] An advantage of FR is that its systemic toxicity is less than that of RA.[5,6] The cytotoxicity of FR is due mainly to its ability to induce apoptosis, although the mechanism has not been fully elucidated.[4] Several studies have shown that, in NB cell lines, FR produced intracellular reactive oxygen species (ROS).[7–9] In addition, FR increases intracellular ceramide, which is known as an inducer of apoptosis, in NB cells.[7,10,11]

Mitogen-activated protein kinases (MAPK) are well-conserved signaling proteins in eukaryotic cells and have essential roles in deciding cell fate.[12,13] Two members of the MAPK family, c-Jun N-terminal kinase (JNK) and p38 MAPK, are activated by various stress stimuli including oxidative stress and chemical agents.[12–14] When activated, they phosphorylate downstream transcription factors of c-Jun and activating transcription factor-2 (ATF-2). Sustained-activation of JNK and p38 MAPK induces cell death.[13,15]

In prostate carcinoma cell lines, FR did not activate p38 MAPK, but it did activate JNK in an ROS-independent manner,[16] and the JNK pathway mediated FR-induced apoptotic signaling.[16,17] It has also been shown that FR activated JNK in A431 epidermoid carcinoma cells.[18] It is not known, however, whether FR activates JNK and p38 MAPK signaling in NB cells, or whether signaling is essential for FR-induced apoptosis. We examined stress signaling and apoptosis induced by FR in NB cells. We found that FR induced sustained-activation of both JNK and p38 MAPK in NB cells, indicating that JNK and p38 MAPK mediate FR-induced apoptosis. We also examined the relationship between the FR-induced ROS generation and the JNK/p38 MAPK signaling, and found that their activation is ROS-dependent. Moreover, we demonstrated that FR failed to produce intracellular ROS and to activate the kinases in the resistant NB cells, indicating the suppression of FR-induced ROS production and activation of JNK/p38 MAPK is one of the mechanisms of resistance to FR in NB cells.

MATERIAL AND METHODS

Cell culture

Human NB cell lines KP-N-TK[19] and KP-N-SIFA[20] were cultured in RPMI 1640 containing penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated FBS at 37°C in a 5% CO_2 incubator. The medium was changed every 3–4 days. Cells were sub-cultured into new flasks by trypsinization when in a sub-confluent state. FR-resistant cells of KP-N-TK, designated as KP-N-TK (FR-R), were established by culturing parental KP-N-TK cells with increasing concentrations of FR from 0.5–5 μM for 150 days. The cells were then maintained continuously in 5 μM FR.

Antibodies and reagents

Polyclonal antibodies against JNK, p38 MAPK, Thr^{183}/Tyr^{185}-phosphorylated JNK, Thr^{180}/Tyr^{182}-phosphorylated p38 MAPK were purchased from *Cell Signaling Technology* (Beverly, MA). Monoclonal anti-caspase-3 antibody was from BD Biosciences (San Jose, CA). Monoclonal anti-caspase-9 and anti-poly (ADP-ribose) polymerase (PARP) antibodies were obtained from Oncogene Research Products (San Diego, CA). FR (Toronto Research

Abbreviations: AA, L-ascorbic acid; CM-H_2DCFDA, 5(-6)-chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate; FR, fenretinide; JNK, c-Jun N-terminal kinase; NB, neuroblastoma; p38 MAPK, p38 mitogen-activated protein kinase; PARP, poly (ADP-ribose) polymerase; ROS, *reactive oxygen species*; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

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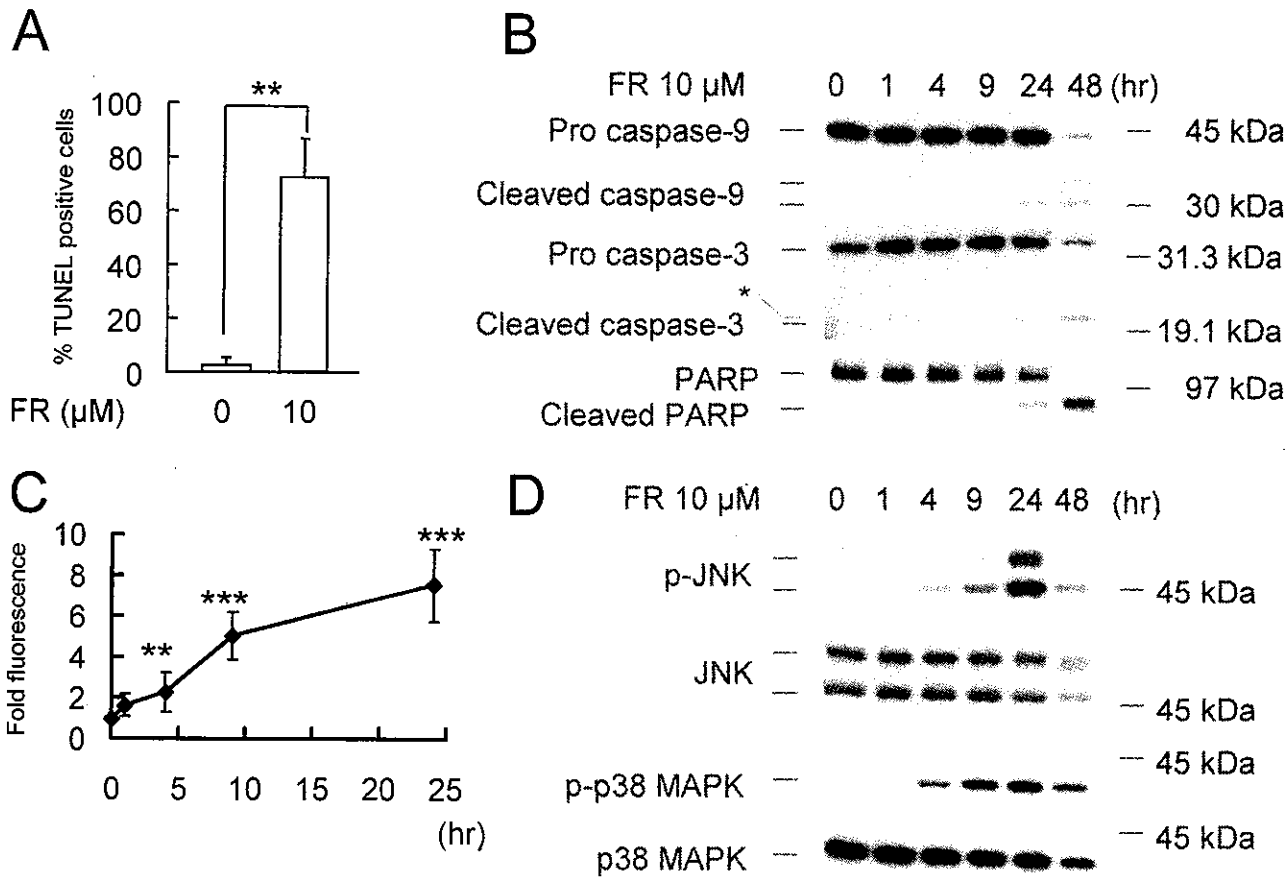

FIGURE 1 – Fenretinide-induced apoptosis, intracellular ROS accumulation and activation of JNK and p38 MAPK in KP-N-TK cells. (*a*) TUNEL assay. Cells were incubated with 10 μM FR or DMSO vehicle for 48 hr. Harvested cells were fixed as described in Material and Methods. After the TdT reaction with FITC labeling, cells were analyzed with a flow cytometer, and the percentage of TUNEL-positive cells was determined (mean + SD, $n = 3$). $^{**}p < 0.01$ (Student's *t*-test). (*b*) Time-course of cleavages of caspase-9, caspase-3 and PARP. Cells were treated with 10 μM FR for the times indicated. Lysates were prepared and immunoblotted for anti-caspase-9, anti-caspase-3 or anti-PARP antibodies. This is representative of 3 independent experiments. The band indicated by an asterisk is a non-specific band. (*c*) Time-course of intracellular ROS accumulation. Cells were incubated with 10 μM FR for the times indicated. CM-H_2DCFDA was added for 2 hr before cell harvesting. Flow cytometric analysis was carried out and the mean fluorescence was calculated (mean + SD, $n = 7$). $^{**}p < 0.01$, $^{***}p < 0.001$ (compared to control, Student's *t*-test) (*d*) Time-course of the activation of JNK and p38 MAPK. Cells were incubated with 10 μM FR for the times indicated. Immunoblotting was carried out using anti-phospho (p)-JNK, anti-JNK, anti-p-p38 MAPK or anti-p38 MAPK. This is representative of 3 independent experiments.

Chemicals, North York, Canada) was dissolved in dimethyl sulfoxide (DMSO) and stored at −70°C in the dark. L-Ascorbic acid (AA) (Wako Pure Chemical Ind., Osaka, Japan) was dissolved in distilled water and stored at −20°C. 5(-6)-Chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Molecular Probes, Eugene, OR) was freshly prepared in DMSO before use. The final concentration of DMSO was <0.2% in all experiments.

Western blotting

Cells (5×10^6) were seeded onto 100-mm dishes. When 50–60% confluence was achieved, cells were treated with 10 μM FR at 37°C with or without pretreatment of 400 μM AA for 12 hr. After the indicated periods, cells were washed once with ice-cold PBS. Floating cells were also collected. Cells were solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate). Protein concentration was determined using a DC protein assay (Bio-Rad Laboratories, Hercules, CA). Cell lysates were electrophoresed on SDS-polyacrylamide gels, and then transferred to a PVDF membrane. The membrane was blocked in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) and 5% nonfat skim milk, and subsequently probed with the primary antibody. The blots were washed in TBS-T and treated with the appropriate secondary antibodies (Amersham, Arlington, IL), and then analyzed using the ECL chemiluminescence system (Amersham).

Apoptosis assay

Apoptosis was determined using the MEBSTAIN Apoptosis Detection Kit Direct (Medical & Biological Laboratories Co., Nagoya, Japan) according to the manufacturer's protocol. In brief, cells (2×10^6) were plated onto 60-mm dishes. At 50–60% confluence, cells were treated with 10 μM FR at 37°C with or without preincubation with 400 μM AA for 12 hr. After 48 hr, cells were harvested, washed with PBS, and then fixed with 4% paraformaldehyde for 30 min at 4°C. Subsequently, cells were permeabilized with 70% ethanol for more than 30 min at −20°C, and incubated with the mixture of TdT and FITC-conjugated dUTP for 1 hr at 37°C. The cells were analyzed with a FACS Calibur flow cytometer (Nippon Becton Dickinson Co., Tokyo, Japan) and the number of the TUNEL (TdT-mediated dUTP-biotin nick end labeling)-positive cells was calculated using Cell Quest software (Nippon Becton Dickinson Co.).

The cleavages of caspase-9, caspase-3 and PARP were also detected by Western blotting as described above.

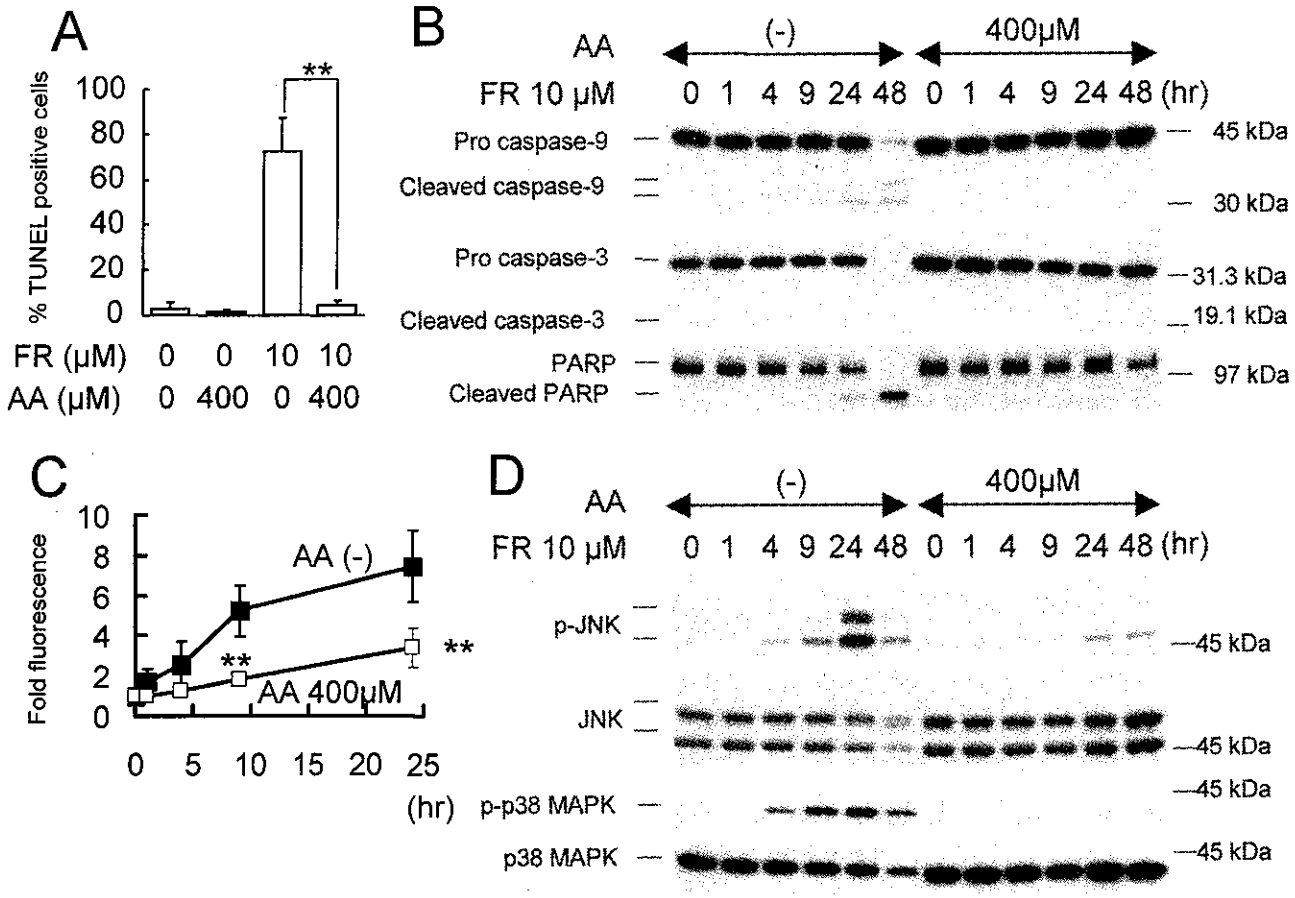

FIGURE 2 – L-Ascorbic acid (AA) inhibits FR-induced apoptosis, ROS accumulation and activation of JNK and p38 MAPK in KP-N-TK cells. Cells were treated with 10 μM FR with or without pretreatment with 400 μM AA for 12 hr. (*a*) TUNEL assay. Cells were incubated with FR or DMSO vehicle for 48 hr. TUNEL assay was carried out as described in Figure 1 (mean + SD, $n = 3$). $^{**}p < 0.01$ (Student's *t*-test). (*b*) Time-course of cleavages of caspase-9, caspase-3 and PARP. Cells were incubated with FR for the times indicated. Immunoblot analysis was carried out as described in Figure 1. This is representative of 3 independent experiments. (*c*) Time-course of intracellular ROS accumulation. Flow cytometric analysis using CM-H_2DCFDA was carried out as described in Figure 1 (mean + SD, $n = 4$). $^{**}p < 0.01$ (Student's *t*-test, compared to no pretreatment of AA). (*d*) Time-course of activation of JNK and p38 MAPK. Cells were incubated with FR for the times indicated with or without pretreatment with AA. Western blot analysis was carried out as described in Figure 1. This is representative of 3 independent experiments.

Determination of intracellular ROS

The intracellular concentration of ROS was measured using CM-H_2DCFDA as a probe. This probe is a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the non-fluorescent polar derivative 2′,7′-dichlorofluorescin and thereby trapped within the cells. In the presence of a proper oxidant, 2′,7′-dichlorofluorescin is oxidized to highly fluorescent 2′,7′-dichlorofluorescein. Cells were plated onto 6-well dishes (5×10^5 cells /well) and treated with 10 μM FR with or without pretreatment of 400 μM AA as above. Two hours before cell harvest, 5 μM CM-H_2DCFDA was added to the cells. After the indicated periods, the medium was removed; cells were washed once with PBS, harvested, and suspended in PBS. The cells were immediately analyzed with a FACS Calibur flow cytometer; the excitation and emission wavelengths were at 488 nm and 530 nm, respectively. The mean fluorescence of 1×10^4 cells per sample was calculated using Cell Quest software.

RESULTS

Fenretinide induces apoptosis, intracellular ROS production and sustained-activation of JNK and p38 MAPK in KP-N-TK NB cells

We first studied FR-induced apoptosis in KP-N-TK cells. After 48 hr incubation with 10 μM FR, up to 70% of the cells were TUNEL-positive (Fig. 1*a*). The DMSO vehicle did not induce apoptosis (Fig. 1*a*). Cleavages of caspase-9, caspase-3 and PARP appeared 24 hr after the treatment, and progressed at 48 hr (Fig. 1*b*). We then studied the intracellular ROS production by FR in KP-N-TK cells. The fluorescence of CM-H_2DCFDA increased gradually during 24 hr of incubation with 10 μM FR (Fig. 1*c*). DMSO vehicle alone did not increase the fluorescence (data not shown).

Fenretinide induced sustained-activation of both JNK and p38 MAPK in KP-N-TK cells (Fig. 1*d*). The phosphorylation of JNK and p38 MAPK was observed from 4–48 hr after treatment with 10 μM FR, and peaked at 24 hr after treatment. The total amounts of JNK and p38 MAPK decreased at 48 hr. Short incubation with FR from 15 min (data not shown) and 1 hr (Fig. 1*d*) did not activate these kinases. The DMSO vehicle alone also did not alter their activation (data not shown).

L-Ascorbic acid suppresses fenretinide-induced apoptosis, intracellular ROS accumulation and activation of JNK and p38 MAPK

KP-N-TK cells were pretreated with 400 μM of the antioxidant AA and then incubated with 10 μM FR for 48 hr. Pretreatment with AA almost completely blocked FR-induced apoptosis (Fig. 2*a*). Fenretinide-induced processing of caspase-9, caspase-3 and PARP was also suppressed in the presence of AA (Fig. 2*b*).

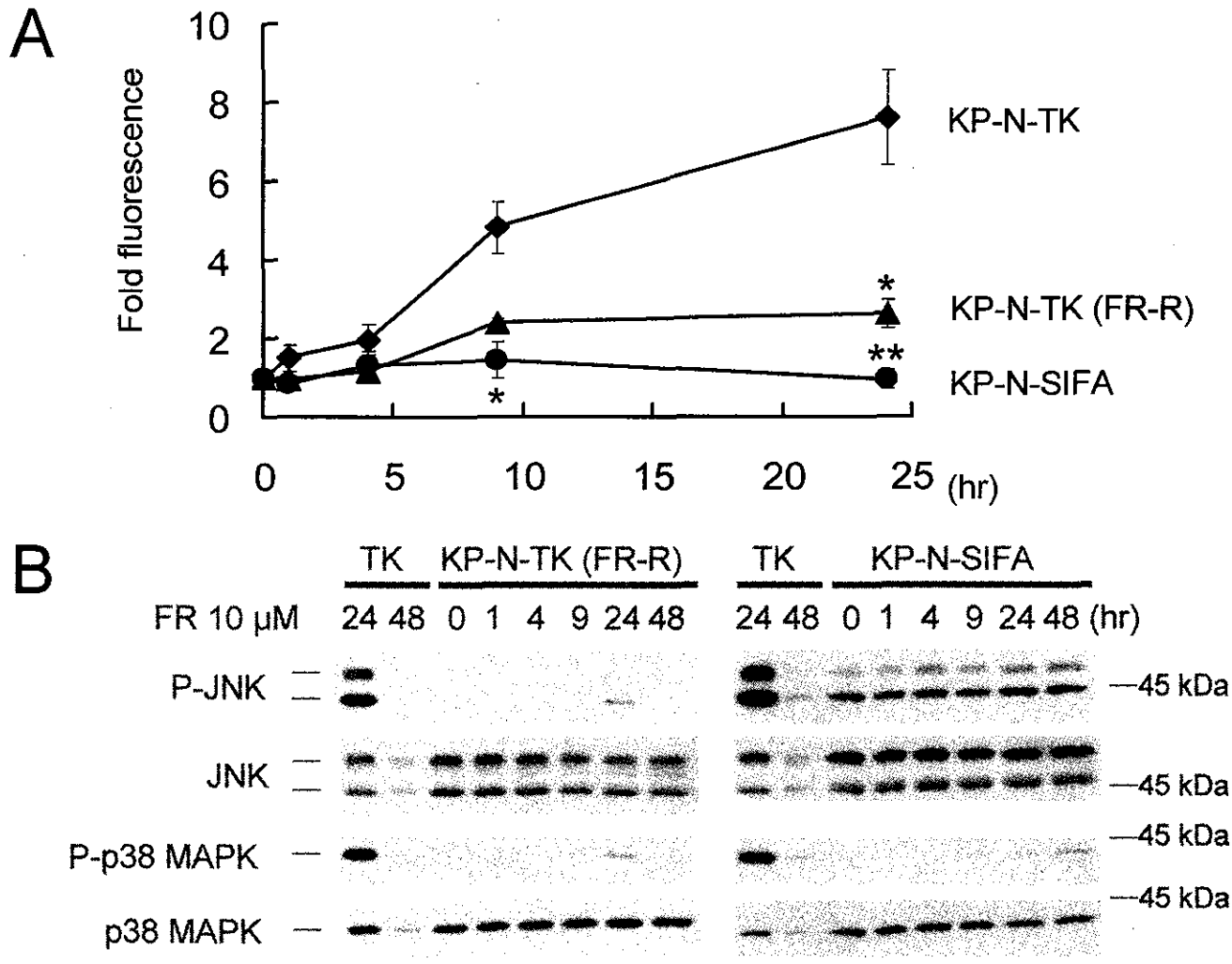

FIGURE 3 – Fenretinide-induced ROS accumulation and activation of JNK and p38 MAPK is suppressed in FR-resistant KP-N-TK (FR-R) and KP-N-SIFA cells. (*a*) Time-course of intracellular ROS. Cells were incubated with 10 μM FR for the times indicated. CM-H_2DCFDA was added for 2 hr before cell harvesting. Flow cytometric analysis was carried out and the mean fluorescence was calculated (mean ± SD, $n = 3$). $*p < 0.05$, $**p < 0.01$ (compared to KP-N-TK, Student's *t*-test). (*b*) Time-course of activation of JNK and p38 MAPK. Cells were treated with 10 μM FR for the times indicated. Immunoblotting was carried out as described above. This is representative of 3 independent experiments.

Furthermore, preincubation of AA suppressed FR-induced intracellular ROS accumulation in KP-N-TK cells (Fig. 2*c*). To determine whether FR-induced activation of JNK and p38 MAPK is ROS-dependent, we compared the phosphorylation of JNK and p38 MAPK induced by FR with or without preincubation with AA in KP-N-TK cells. In the presence of AA, FR-induced activation of JNK and p38 MAPK was suppressed markedly (Fig. 2*d*).

Fenretinide-induced intracellular ROS accumulation and activation of JNK and p38 MAPK is decreased in fenretinide-resistant NB cell lines

To investigate the mechanism of resistance to FR in NB cells, we generated a FR-resistant KP-N-TK cell line, KP-N-TK (FR-R) cells. KP-N-TK (FR-R) was highly resistant to FR even at a concentration of 10 μM FR (data not shown). When KP-N-TK (FR-R) was incubated in FR-free medium, its resistance to FR was preserved (data not shown). KP-N-SIFA cells exhibited complete resistance to 10 μM FR (data not shown).

To determine whether FR-induced intracellular ROS production was altered in the resistant NB cells, we treated FR-resistant KP-N-TK (FR-R) cells, KP-N-SIFA cells and FR-sensitive parental KP-N-TK cells with 10 μM FR and compared their intracellular ROS levels. Less FR-induced intracellular ROS were generated in the 2 resistant cell lines than in the sensitive KP-N-TK cells (Fig. 3*a*).

FR induced little phosphorylation of the kinases in the resistant KP-N-TK (FR-R) and KP-N-SIFA cells (Fig. 3*b*). Although phosphorylated JNK was present in KP-N-SIFA cells even in the absence of FR, FR did not increase its level during 48 hr.

DISCUSSION

FR is known to induce apoptosis in NB cells but the mechanism is not clear. Our results demonstrate that FR induced sustained-activation of JNK and p38 MAPK signaling in an ROS-dependent manner, and finally induced apoptosis in NB cells. In FR-resistant NB cells, FR failed not only to generate intracellular ROS but also to activate JNK and p38 MAPK signaling. Thus, the alterations in FR-resistant NB cells prevent FR from inducing apoptosis.

ROS are known to induce apoptosis. In KP-N-TK cells, FR induces prolonged production of intracellular ROS. FR-induced ROS generation precedes the processing of caspase-9, caspase-3 and PARP, indicating that ROS generation is upstream of the

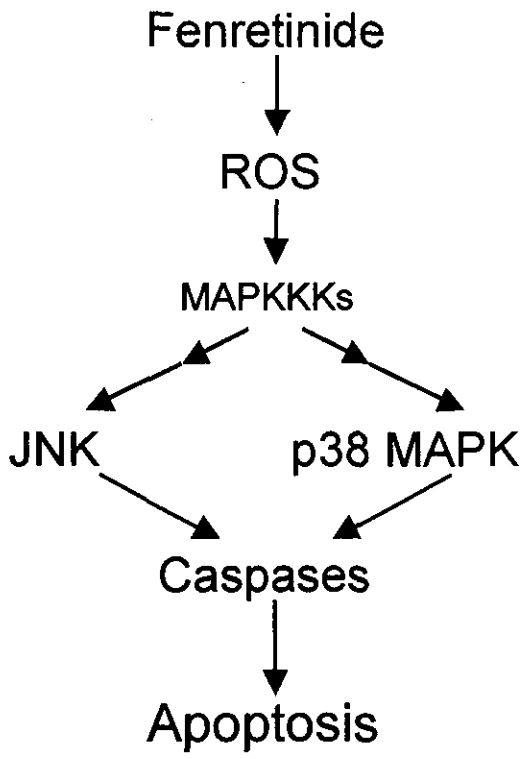

FIGURE 4 – Schematic representation of FR-induced ROS generation, JNK and p38 MAPK signaling and apoptosis in neuroblastoma cells. FR induces sustained-activation of JNK and p38 MAPK signaling in an ROS-dependent manner. The sustained activation of JNK and p38 MAPK activation leads to processing of caspases and apoptosis.

caspase cascade. The antioxidant AA almost completely blocked apoptosis and suppressed FR-induced intracellular ROS accumulation. Our results show that AA has a more complete inhibitory effect on FR-induced apoptosis in NB cells than on FR-induced apoptosis in other NB cell lines (CHLA-90, SMS-LHN, SMS-KCNR[7] and SH-SY5Y[8]).

FR activated both JNK and p38 MAPK in FR-sensitive KP-N-TK NB cells. In KP-N-TK cells, the activation of JNK and p38 MAPK by FR (starting after 4 hr of FR treatment) preceded the cleavages of caspase-9, caspase-3 and PARP (starting after 24 hr of FR treatment), indicating that the JNK and p38 MAPK signaling pathways are upstream of the caspase cascades, as reported in the LNCap and PC3 prostate carcinoma cell lines.[16,17] Furthermore, FR-induced activation of JNK and p38 MAPK is sustained for 48 hr, suggesting the possible role of the signaling in FR-induced apoptosis.[13–15] Interestingly, in prostate carcinoma LNCap cells, FR activated JNK but not p38 MAPK.[16]

Our finding that FR-induced activation of JNK and p38 MAPK in NB cells was ROS-dependent is supported by the finding that AA suppressed FR-induced activation of JNK and p38 MAPK signaling in KP-N-TK cells. In prostate carcinoma cells, Chen *et al.*[16] found that FR-induced JNK activation was not affected by the antioxidant *N*-acetyl-L-cysteine (NAC), indicating that FR activated JNK independent of ROS. They did not, however, determine whether NAC suppressed the accumulation of FR-induced intracellular ROS. In our present study, AA clearly reduced FR-induced intracellular ROS accumulation.

JNK and p38 MAPK pathways have been shown to be involved in ROS-induced apoptosis.[13,14] When ROS production is low, JNK and p38 MAPK are only transiently activated, and cells will survive.[13,14] On the contrary, high ROS production induces sustained-activation of JNK and p38 MAPK, and finally leads to cell death.[13,14] The quantity and duration of oxidative stress will determine which MAPK kinase kinases (MAPKKK) are activated, which will determine the activation pattern of JNK and p38 MAPK, which, in turn, will decide cell fate.[13,14]

ROS activate various signaling pathways other than MAPKKK, including c-Abl tyrosine kinase.[14] c-Abl activates both JNK and p38 MAPK in the response to DNA damage[22] and also mediates ROS-induced apoptosis under some conditions.[23] The c-Abl-p38 MAPK-p73 pathway is thought to be essential for apoptosis induced by chemotherapeutic agents.[24] This pathway might be also involved in fenretinide-induced apoptosis, although we did not examine the expression of p73 in these cells.

FR failed to induce intracellular ROS accumulation in the FR-resistant KP-N-TK (FR-R) and KP-N-SIFA NB cell lines, indicating that suppression of the ROS-productive system is responsible for the resistance to FR in NB cells. Similarly, FR produced less free radicals in FR-resistant SH-SY5Y cells.[8] ROS production in the FR-resistant A2780 ovarian cancer cell line was not significantly different from that in the FR-sensitive parental cells.[21] In FR-resistant NB cells, FR failed to activate JNK and p38 MAPK signaling. This result further supports the hypothesis that FR activates JNK and p38 MAPK signaling in an ROS-dependent manner. It also indicates that sustained-activation of JNK and p38 MAPK is responsible for FR-induced apoptosis. It is of interest to know whether these FR-resistant cells are also resistant to other cytotoxic agents, especially ROS-producing ones such as cisplatin. In our preliminary results, FR-resistant KP-N-SIFA cells were the most sensitive to cisplatin among the cell lines used in our study (data not shown). KP-N-TK cells, as well as KP-N-TK (FR-R) cells, were resistant to 20 μM cisplatin (data not shown). These data suggest that the mechanisms of FR resistance and cisplatin resistance are different.

In conclusion, we demonstrated for the first time that FR induces sustained activation of JNK and p38 MAPK in an ROS-dependent manner in FR-sensitive NB cells (Fig. 4), but not in FR-resistant cells. Moreover, our results raise the possibility that sustained-activation of the stress signaling pathway mediates FR-induced apoptosis. Our results also show for the first time that suppression of the intracellular ROS productive system and the downstream JNK/p38 MAPK pathways are related to FR-resistance in NB cells.

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Clinical Significance of a Highly Sensitive Analysis for Gene Dosage and the Expression Level of *MYCN* in Neuroblastoma

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Fukuoka, Japan

Background: The amplification of the *MYCN* gene is one of the most powerful adverse prognosis factors in neuroblastoma, but the clinical significance of an enhanced expression of *MYCN* remains controversial. To reassess the clinical implications of *MYCN* amplification and expression in neuroblastoma, the status of amplification and the expression level of the *MYCN gene* of primary neuroblastoma samples were analyzed using highly sensitive analyses.

Methods: Using a quantitative polymerase chain reaction (PCR) method (TaqMan), the gene dosages (*MYCN/p53*) of 66 primary neuroblastoma samples were determined. In all 66 samples, the status of *MYCN* amplification has been determined previously by the Southern blotting method. Of the 54 samples with a single copy of *MYCN* based on the Southern blotting method, 23 samples were analyzed for *MYCN* amplification using the fluorescence in situ hybridization (FISH) method. The expression levels (*MYCN/GAPDH*) of 56 samples were determined by a quantitative reverse transcriptase (RT)-PCR method.

Results: Of the 54 samples with a single copy of *MYCN* based on the Southern blotting method, 46 samples showed *MYCN* gene dosages of less than 2.0, whereas the remaining 8 samples with dosages of more than 2.0 were tumors from patients with advanced-stage disease. The results of FISH supported the fact that these 8 samples contained a small number of *MYCN*-amplified cells. The cases of *MYCN* gene dosages of more than 2.0 were significantly associated with all other unfavorable prognostic factors (an age of >1 year at diagnosis [$P < .0001$], nonmass screening [$P = .0003$], advanced stage [$P < .0001$], diploid or tetraploid [$P < .0001$], and a Shimada unfavorable histology [$P < .0001$]). *MYCN* gene dosages of more than 2.0 were significantly associated with a high expression of *MYCN* ($P = .0459$). However, the expression level of *MYCN* was not significantly associated with any other prognostic factors.

Conclusions: Quantitative PCR may thus be a useful modality for performing a highly sensitive and accurate assessment of the amplification and expression levels of the *MYCN* gene. In particular, the combination of the quantitative PCR system and the FISH method is considered to be a highly effective method for evaluating the status of *MYCN* amplification. In this highly sensitive analysis, *MYCN* amplification (*MYCN/p53* $\geq$ 2.0) was reconfirmed to be a strongly unfavorable factor, whereas the expression level of *MYCN* does not appear to be an independently significant prognosis factor. *J Pediatr Surg 39:63-68.*

INDEX WORDS: Neuroblastoma, *MYCN*, quantitative polymerase chain reaction, FISH.

NEUROBLASTOMA is a tumor derived from neural crest origin, which arises in the adrenal medulla or paraspinal sympathetic ganglia. The prognosis in neuroblastoma tends to vary greatly, and many studies have found both clinical and biological factors to be closely correlated with the outcome.[1,2] To select the optimal treatment according to the degree of malignancy of neuroblastoma, it is essential to accurately and rapidly identify any genetic abnormalities associated with the prognosis using several molecular biological methods. The amplification of the *MYCN* gene is strongly associated with rapid tumor progression.[3,4] An amplification of the *MYCN* gene occurs in approximately 25% of primary tumors, and this factor is known to be one of the most unfavorable prognostic factors in neuroblastoma.[3,4] We assume that an enhanced expression consequent to the gene amplification of proto-oncogene contributes to tumorigenesis. Regarding the *MYCN* gene, it is easy to consider that the amplification of *MYCN* gene results in an enhanced expression of *MYCN*, which activates the transcription of genes associated with the cell proliferation.[5,6] However, the clinical significance of *MYCN* expression in children with neuroblastoma remains controversial.[7-11]

The amplification of the *MYCN* status has been mainly analyzed for the whole tumor using the Southern blotting method, but this method is not able to detect intratumor heterogeneity. We previously reported our findings for a

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highly sensitive analysis for *MYCN* amplification in neuroblastoma based on the FISH (fluorescence in situ hybridization) method.[12] Furthermore, we reported preliminarily the quantitative polymerase chain reaction (PCR) method (TaqMan) to be useful as a quick and accurate modality for evaluating for the status of *MYCN* amplification in 25 neuroblastoma samples.[13]

In the current study, the status of *MYCN* amplification in more neuroblastoma samples was evaluated using the quantitative PCR method and the FISH method to reassess the clinical implication of *MYCN* amplification in neuroblastoma. Furthermore, the expression level of *MYCN* was analyzed also by the quantitative PCR method to elucidate the correlation between *MYCN* expression and the biology of neuroblastoma.

MATERIALS AND METHODS

Clinical Data of Patients and Biological Data of Neuroblastoma Samples

Neuroblastoma in patients evaluated at the Department of Pediatric Surgery, Kyushu University was diagnosed and staged according to the International Neuroblastoma Staging System (INSS).[14] Sixty-six frozen tumor samples were obtained from untreated neuroblastoma patients. The characteristics of the patients were shown to be as follows: the sex of the patients was 39 boys and 27 girls, and the age at diagnosis ranged from 19 days after birth to 11 years of age. Of the 66 cases, 25 cases were diagnosed in patients greater than 1 year of age, whereas the remaining 41 cases were diagnosed in patients younger than 1 year of age. Thirty-six patients were identified by a neuroblastoma mass screening system. Of the 66 samples, 39 were tumors that were stage 1, 2, or 4S, whereas 9 were stage 3, and 18 were stage 4. Fifty-three patients are still alive, of whom, 5 patients are still under treatment, whereas 13 patients have died of the disease. The follow-up period after treatment ranged from 1 month to 12 years. In all 66 tumors, the *MYCN* amplification status was analyzed by the quantitative PCR system, and the expression level of *MYCN* was analyzed by the quantitative RT-PCR system in the 56 tumors. In all 66 samples, the status of *MYCN* amplification was also previously determined by the Southern blotting method.[15] In 23 of 54 cases with a single copy identified by Southern blotting, the *MYCN* amplification status was examined using the FISH methods. DNA ploidy was examined previously using flow cytometry[16] in 46 cases. Thirty-one cases were triploid, whereas 15 cases were diploid or tetraploid. Regarding the histologic findings, 64 cases were classified based on the Shimada classification.[17] Forty-five cases showed a favorable histology, whereas the remaining 19 cases showed an unfavorable histology.

DNA or RNA Extraction and cDNA Synthesis

DNA was extracted from the frozen tumor samples using proteinase K and phenol. Isogen LS (Nippon Gene, Osaka, Japan) was used to extract total RNA, and reverse transcription (RT) was performed with a First-strand cDNA synthesis kit (Amersham Pharmacia, Uppsala, Sweden) using random hexanucleotide primers.

Quantitative PCR (TaqMan)

As described previously, the *p53* gene was used as an internal control gene to obtain the gene dosage (*MYCN/p53*).[13] The *p53* gene is a tumor suppresser gene in which mutations or deletions are found in a variety of malignant tumors. However, no aberration of the *p53* gene in neuroblastoma has ever been found, and the gene status in neuroblastoma is known to be stable.[18] The corrected gene dosage of the *MYCN* gene was obtained based on the assumption that the mean gene dosage of 20 normal individual lymphocytes was 1.00. The mean ± 2 SD of gene dosage of 20 normal individual lymphocytes was 1.00 ± 0.58. In this study, we evaluated that the *MYCN*-amplified cells apparently present in the samples with a corrected gene dosage (*MYCN/p53*) of more than 2.0. The information on the *MYCN* gene and the *p53* gene sequences was obtained from a GenBank database search. The primers and TaqMan probes for the *MYCN* gene and the *p53* gene were designed to be located on intron 2 of *MYCN* and exon 1 of the *p53* gene using the application-based primer design software Primer Express (Applied Biosystems, Foster City, CA). The sequences of the PCR primers and TaqMan probes were as follows: *MYCN:* forward primer 5′-CCC AGC GTG GTA GTC AAT GA-3′, reverse primer 5′-TTA ATG ACA AAG CCA TAA TCC ACA G-3′, TaqMan probe 5′-AGA ATG CGC ACA TGA TGC TAC ACG TTT CT-3′; *p53:* forward primer 5′-GCC CTT ACT TGT CAT GGC GA-3′, reverse primer 5′-ATC CCA CAA CCC CTG CG-3′, TaqMan probe 5′-TGT CCA GCT TTG TGC CAG GAG CC-3′. Quantitative PCR was performed in a final volume of 25 μL, and each sample was analyzed in duplicate. Each reaction mixture contained 0.1 pmol/μL TaqMan probe, 0.2 pmol/μL each primer, 1x TaqMan PCR master mix, and 10 to 50 ng DNA. Thermal cycling was started with a 2 minute incubation at 50°C, followed by a first denaturation step of 10 minutes at 95°C, and then 40 cycles of 2-step PCR consisting of 95°C for 5 seconds and 60°C for 1 minute. The quantification of the *MYCN* gene was achieved by means of the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Genomic DNA from one neuroblastoma with 90 copies of *MYCN* by Southern blotting method was serially diluted to establish the calibration curve.

Quantitative RT-PCR (TaqMan)

The primer and TaqMan probe for *MYCN* mRNA were designed to be located on exon 2-3, thereby avoiding the amplification contaminating genomic DNA. *GAPDH* was used as an internal control gene to analyze the *MYCN* gene expression (*MYC/GAPDH*). The sequences of the PCR primers and TaqMan probe were as follows: *MYCN:* forward primer 5′-GAC CAC AAG GCC CTC AGT ACC-3′, reverse primer 5′-TGA CCA CGT CGA TTT CTT CCT-3′, TaqMan probe 5′-CCG GAG AGG ACA CCC TGA GCG A-3′. PCR primer and TaqMan probe for *GAPDH* were purchased from ABI as a kit of TaqMan GAPDH Control Regent and Predeveloped TaqMan Assay Regents Control Kit. The measurement of *MYCN* cDNA was based on 2 reporter dyes, namely, FAM for *MYCN* and VIC for *GAPDH*. The quantitative RT-PCR system was performed in the same manner as that for the quantitative PCR. The corrected expression level of *MYCN* (*MYCN/GAPDH*) was obtained based on the assumption that the value of one primary tumor sample (12 copies of *MYCN* by Southern blotting) was 1.00.

FISH Analysis of the MYCN Gene

Fresh tumor single cells were suspended in potassium chloride. The nuclei were denatured immediately before hybridization in 70% formamide and 2 times standard saline citrate (SSC) at 75°C for 2 minutes and then were dehydrated through ethanol. The hybridization buffer, which contained 10 ng aliqour of the *MYCN* probe was denatured for 5 minutes at 75°C, chilled on ice, and then applied to the slide. Hybridization was performed overnight at 37°C. After the slide was washed by formamide, SSC, and Triton, the nuclei were counterstained with 30 μL of DAPI containing 5 μL of antifade solution. The signals representing the *MYCN* gene were countered for 100 cells on one slide.

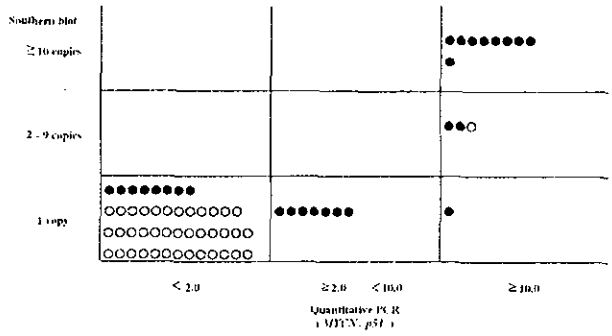

Fig 1. The comparison between the quantitative PCR method and Southern blotting method for the status of *MYCN* amplification in 66 primary neuroblastomas. ●, stage 3, 4; ○, stage 1, 2, 4S.

The images were photographed using a fluorescence microscope (Olympus, BX60, Tokyo, Japan) and Provia 400 (Fuji, ISO 400, Tokyo, Japan).

Statistical Analysis

Fisher' Exact test was used to test the association between *MYCN* amplification (*MYCN/p53* ≥ 2.0) or no amplification (*MYCN/p53* < 2.0) and other prognostic factors. The expression levels of *MYCN* (*MYCN/p53*) in the subgroups were represented by Percentile (50% [25%, 75%]). A comparison of the gene dosage and expression in relation to clinical and genetic parameters was made using Mann-Whitney U test.

RESULTS

The Gene Dosages of MYCN by the Quantitative PCR Method

Regarding the status of *MYCN* amplification, the findings of a comparison between the quantitative PCR method and the Southern blotting method are shown in Fig 1.

Of the 54 samples with a single copy of *MYCN* based on the Southern blotting method, 46 samples showed the corrected gene dosage (*MYCN/p53*) to be less than 2.0, whereas the remaining 8 samples with more than 2.0 had tumors from patients with an advanced stage of disease (stages 3, 4). Of the 8 samples with a dosage of more than 2.0, 3 patients died of the disease.

In 23 of 54 cases with a single copy of *MYCN* based on the Southern blotting method, the status of *MYCN* amplification was also examined by the FISH method (Fig 2). Eighteen of the samples were tumors from the patients with a nonadvanced stage, whereas the remaining 5 samples were those from patients with a advanced stage. The patients with a gene dosage of more than 2.0 (*MYCN/p53* ≥ 2.0) and a single copy of *MYCN* by the Southern blotting method, only 2 patients had an advanced stage. Furthermore, the FISH analysis showed that these 2 cases (case 1 and 2) contained a small number of *MYCN* amplified cells at rates of 15% and

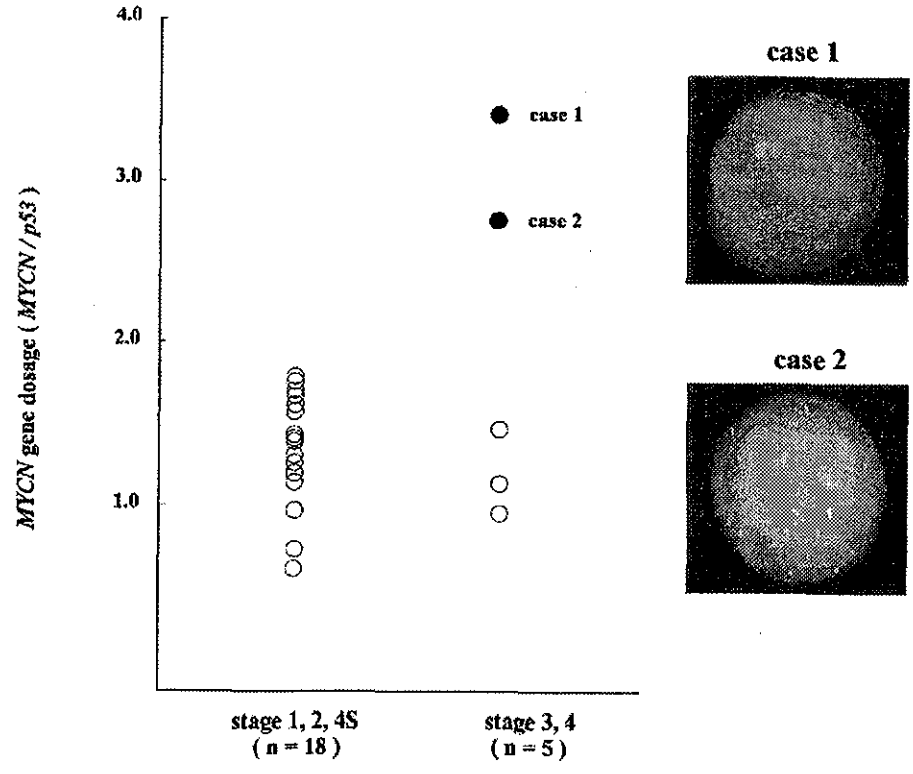

Fig 2. The gene dosage of *MYCN* based on the quantitative PCR and the status of *MYCN* amplification by the FISH method in 23 neuroblastomas with a single copy of *MYCN* based on the Southern blotting method. The 2 cases (cases 1 and 2) contained a small number of *MYCN*-amplified cells at rates of 15% and 29% of the cells with a nuclei, respectively. ●, *MYCN*-amplified cell (+) by FISH; ○, *MYCN*-amplified cell (−) by FISH.

Table 1. Gene Dosage of *MYCN* in Relation to Clinical and Biological Prognostic Factors

Category	Number	Gene Dosage of *MYCN* (*MYCN/p53*)		*P* Value*
		<2.0	≥2.0	
Age				
<1 year of age	41	37 (90.2%)	4 (9.8 %)	<.0001
≥1 year of age	25	9 (36.0%)	16 (64.0%)	
Mass screening				
Mass positive	36	32(88.9%)	4 (11.1%)	.0003
Sporadic	30	14 (46.7%)	16 (53.3%)	
Stage				
Stage 1,2,4S	39	38 (97.4%)	1 (2.6 %)	<.0001
Stage 3,4	27	8 (29.6%)	19 (70.4%)	
DNA ploidy				
Aneuploid	31	30 (96.6%)	1 (3.4 %)	<.0001
Diploid or tetraploid	15	4 (26.7%)	11 (73.3%)	
Shimada				
Favorable	45	41 (91.1%)	4 (8.9 %)	<.0001
Unfavorable	19	5 (26.3%)	14 (73.7%)	

**P* value was determinated by Fisher's Exact test.

29% of cells with a nuclei, respectively. These findings suggested that these samples showed intratumor heterogeneity with respect to *MYCN* amplification.

In 9 cases of more than 2 copies of *MYCN* based on the Southern blotting method, the corrected *MYCN* gene dosages by the quantitative PCR were all more than 10.0 (Fig 1). In the majority of these cases, the analytic value based on the quantitative PCR was shown to be a higher than that based on a Southern blotting analysis.

The relationship between the *MYCN* gene dosage and the known prognostic factors (age, mass screening, clinical stage, DNA ploidy, Shimada classification) is shown in Table 1. The cases of a gene dosage of more than 2.0 were strongly associated with an age of more than 1 year at diagnosis ($P < .0001$), nonmass screening ($P = .0003$), advanced stage ($P < .0001$), diploid or tetraploid ($P < .0001$), and a Shimada unfavorable histology ($P < .0001$), which are all unfavorable factors.

The Expression Level of MYCN by the Quantitative RT-PCR Method

The dot graph of gene dosages and expression level of *MYCN* gene in 56 neuroblastomas is shown in Fig 3. As for the expression level of *MYCN* (*MYCN/ GAPDH*), the median value was 0.295. Of 14 cases of *MYCN* amplification (*MYCN/p53* ≥ 2.0), 10 cases showed to be more than the median value (0.295). On the other hand, the cases of no amplification (*MYCN/p53* < 2.0) showed a variety of expression levels of the *MYCN* gene.

The relationship between the *MYCN* gene expression level and prognostic factors are shown in Table 2. The level of *MYCN* expression in cases of *MYCN* amplification (*MYCN/p53* ≥ 2.0) was significantly higher than that of cases of no *MYCN* amplification (*MYCN/p53* < 2.0; $P = .0459$). However, the expression level of *MYCN* was not significantly associated with any other prognostic factors (age, mass screening, clinical stage, DNA ploidy, Shimada classification).

Figure 4 shows the relationship between the *MYCN* gene expression level and age at diagnosis in subsets of tumors with or without *MYCN* amplification. In the cases of no *MYCN* amplification (*MYCN/p53* < 2.0), the *MYCN* expression levels in the samples from patients less than 1 year of age (n = 35, 0.28 [0.06, 1.24]) were significantly higher than those from patients older than 1 year of age (n = 7, 0.02 [0.02, 0.15]; $P = 0.014$). In the cases of no *MYCN* amplification (*MYCN/p53* < 2.0), the majority of patients less than 1 year of age showed an early clinical stage and a good prognosis in spite of an expression level of the *MYCN* gene, whereas the majority of patients older than 1 year of age showed an advanced clinical stage even if the level of *MYCN* expression was very low.

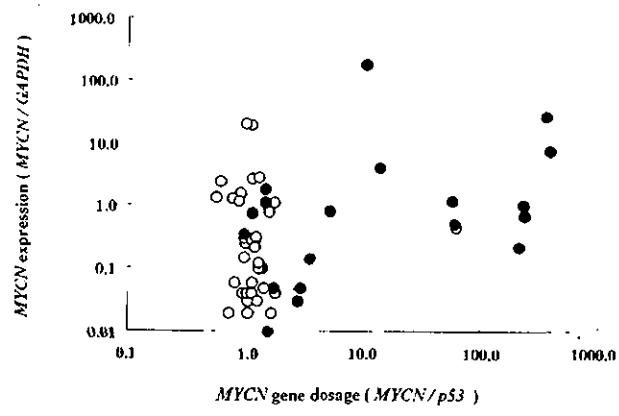

Fig 3. A dot graph of the gene dosages and the expression level of the *MYCN* gene in 56 neuroblastomas. ●, stage 3, 4; ○, stage 1, 2, 4S.

Table 2. *MYCN* Expression in Relation to Clinical and Biological Prognostic Factors

Category	No.	*MYCN/GAPDH* % TILE 50% [25%, 75%]	*P* Value*
Age			
<1 year of age	39	0.31 [0.08,1.24]	.4983
≥1 year of age	17	0.25 [0.03,1.00]	
Mass screening			
Mass positive	34	0.31 [0.10,1.26]	.3739
Sporadic	22	0.24 [0.04,0.95]	
Stage			
Stage1,2,4S	37	0.15 [0.04,1.12]	.0805
Stage 3,4	19	0.78 [0.18,1.53]	
DNA ploidy			
Aneuploid	28	0.22 [0.06,1.14]	.3667
Diploid or tetraploid	9	0.10 [0.03,0.45]	
Shimada			
Favorable	43	0.25 [0.06,1.16]	.4727
Unfavorable	13	0.52 [0.05,1.19]	
MYCN/p53			
<2.0	42	0.19 [0.04,1.12]	.0459
≥2.0	14	0.75 [0.28,3.19]	

*P value was determinated by Mann-Whitney U test.

DISCUSSION

The amplification of the *MYCN* gene is the most powerful unfavorable prognostic factor in neuroblastoma.[3,4] In the Study Group of Japan for Advanced Neuroblastoma (JANB), 2 chemotherapeutic regimens for advanced neuroblastoma have been designed based on the *MYCN* amplification status (more than 10 copies of *MYCN* or less than 10 copies of *MYCN*) since 1991.[19] As a result, it is essential for the treatment of neuroblastoma to accurately and rapidly identify the status of *MYCN* amplification. The quantitative analysis of the *MYCN* gene has so far been mainly analyzed for whole tumors by the Southern blotting method, but this method is not able to detect intratumor heterogeneity. The FISH method is able to evaluate the status of *MYCN* amplification in individual neuroblastoma cells; however, it is difficult to determine the copy number of the *MYCN* oncogene using the FISH method. The quantitative PCR system is a new technique,[20] which is able to overcome the demerits of both the FISH and the Southern blot methods.

In the current study, the combined analysis of the quantitative PCR and the FISH method suggested that approximately 15% (8 of 54) of all cases with a single copy by the Southern blotting method might have a small number of *MYCN* amplified cells in those tumors. The quantitative PCR system may be able to detect a small number of *MYCN* amplified cells, which could not be found by the Southern blotting method. The combination of the quantitative PCR system and the FISH method is considered to be highly effective for evaluating the amplification of the *MYCN* status in neuroblastoma. However, it remains unclear as to which treatment should be selected in tumors with a small number of *MYCN*-amplified cells. Further studies based on this combined analysis should lead to the development of new therapeutic strategies. In addition, the quantitative PCR system is also available for small amounts of samples. Therefore, the quantitative PCR system combined with the microdisection technique[21] is considered to be more effective for evaluating the status of *MYCN* amplification.

As mentioned previously, the clinical significance of *MYCN* expression in children with neuroblastoma remains controversial. Bordow et al[7] reported that in patients older than 1 year of age with *MYCN* nonamplified tumors, high levels of *MYCN* expression correlated with poor outcome. On the other hand, a poor survival rate has been observed in patients older than 1 year with advanced-stage tumors that lack *MYCN* amplification, even if the *MYCN* was expressed at low levels.[22] In addition, several researchers reported that the *MYCN* expression was not predictive of a poor prognosis.[8,10] In the current study, *MYCN* gene dosages of more than 2.0 were significantly associated with a high expression of *MYCN*. However, the patients with no amplification (*MYCN/p53* < 2.0) showed various expression levels of *MYCN* gene. In addition, no statistically significant difference between the *MYCN* expression and other prognostic factors (age, mass screening, DNA ploidy, Shimada classification) could be found. These findings suggest that the only gene dosage of *MYCN* does not always

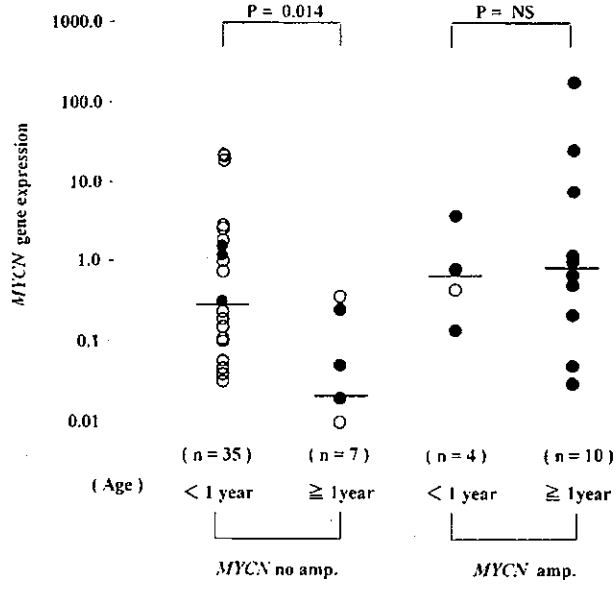

Fig 4. The relationship between the *MYCN* gene expression level and age at diagnosis in the subsets of tumors with or without *MYCN* amplification. *MYCN* amp, *MYCN/p53* ≥ 2.0; *MYCN* no amp, *MYCN/p53* < 2.0. ●, stage 3, 4; ○, stage 1, 2, 4S; bar, 50%tile; NS, not significant.

contribute to the level of *MYCN* expression in neuroblastoma. Furthermore, moderately high levels of *MYCN* expression in patients less than 1 year of age were shown to not be a poor prognostic factor, whereas the low levels of *MYCN* expression in patients greater than 1 year of age were not shown to be a favorable prognostic factor. These results are consistent with the results previously reported by Matsunaga et al[23] who showed the enhanced expression of *MYCN* in cases detected by mass screening.[23] Taken together, the expression level of *MYCN* does not seem to be an independently significant prognostic factor in this highly sensitive analysis.

An enhanced expression of *MYC* has been shown to confer growth potential to cells in vitro as well as in vivo: however, the *MYC* family of oncogenes are also strong inducers of apoptosis in conditions in which their expression is deregulated from mitogenic signaling pathways.[24] Several investigators have reported a correlation between *MYCN* and apoptosis in neuroblastoma in vitro.[25] *MYCN* may thus be considered to play an important role in both the cellular proliferation and apoptosis of neuroblastoma in vivo, and *MYCN* coordinately induces cellular proliferation and apoptosis through different pathways. Further studies on *MYCN*-related proteins should provide an explanation for the mechanism of cellular proliferation and spontaneous regression in neuroblastoma.

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