Ablチロシンキナーゼ阻害剤としてメシル酸イマチニブ（imatinib mesylate，グリベック®），またAPLに対して，全トランス型レチノイン酸（ATRA）はすでに臨床応用されている．固形腫瘍では横紋筋肉腫に見られるPAX3/FKHRやEwing肉腫のEWS/FLI1などのキメラ遺伝子も標的分子としての可能性がある．

従来の抗がん剤では，腫瘍の縮小効果が，早期臨床試験の効果判定として重要であった．しかし，分子標的薬剤の場合，腫瘍の増殖抑制効果や患者のQOL向上も含めて有効性を評価するようになってきた．臨床的有効性を評価するには，薬剤が標的分子に作用していることの証明（proof of target：POT），標的分子に作用することで*in vitro*と*in vivo*ともに，増殖抑制などの効果を示すことの証明（proof of principle：POP），さらに分子標的薬が客観的に臨床症状やQOLの改善などベネフィットにつながることの客観的な証明（proof of efficacy：POE）が最終的に必要となる．

悪性横紋筋肉腫様腫瘍へのゲフィチニブの有用性の検討

我々は，悪性横紋筋肉腫様腫瘍（malignant rhabdoid tumor; MRT）に対するチロシンキナーゼを阻害するゲフィチニブ（gefitinib，イレッサ®）の効果を検討したので紹介する[12]．

MRTは，乳幼児に好発する非常に予後の悪い腫瘍である．組織学的には好酸性封入体の存在が特徴であり，分子生物学的には*hSNF5INI1*遺伝子の異常が認められる．化学療法など治療法が進歩した現在においてもその予後は悪く，4年生存率は非進行例では41.8%であるが，進行例では15.9%である．特に6か月未満発症例の4年生存率は8.8%以下と悲劇的であり[13]，新規治療法の開発が急務である．そこで，MRTに対する分子標的療法の可能性を検討した．

最初にMRTに対する標的分子を検索した．MRT腫瘍組織でのEGFRの発現を確認した報告はなかったが，MRT細胞株にEGFRが発現しているという報告はあった．そこで，我々は，経験した2症例のMRT組織と，それらより樹立したMRT細胞株でのEGFRの発現を検討した．

使用したMRT腫瘍組織は組織学的にMRTと診断された，肝原発のMRT-ANと腎原発のMRT-NSを用いた．また細胞株はこれら2例の腫瘍より樹立されたMP-MRT-AN（以下AN株）とKP-MRT-NS（以下NS株）を使用した．これら細胞株では，*INI1*遺伝子の欠損がみられ，ともに分子生物学的にもMRT細胞株であることが確認された．腫瘍組織におけるEGFRの発現は免疫組織化学法で，細胞株におけるEGFRの発現は間接蛍光抗体法とWestern blot法で検討した．腫瘍組織ではANで瀰漫性にNSでは結節性にEGFRの発現を認め，細胞株ではAN株の90%とNS株の40%にEGFR陽性細胞を認めた（図2）．また，Western blot法でもEGFR蛋白の発現を確認した．これらの結果から，MRTにおいてEGFRが分子標的療法の標的分子となりうることが期待できた．そこで，ゲフィチニブの抗腫瘍効果を*in vitro*と*iv vivo*で評価し，MRTの治療へのゲフィチニブの有用性を検討した．

次に，MRTのEGFRがリン酸化し，ゲフィチニブがEGFRのリン酸化を抑制することが可能かを検討した．リン酸化EGFRの検出は抗EGFR抗体を用いた免疫沈降法を用いて，EGFR蛋白を抽出し，抗チロシンリン酸化抗体を用いたWestern blot法で評価した．無血清下で，MRT細胞株ではリン酸化EGFRは検出できなかったが，EGFを添加すると強くリン酸化された．しかし，1 μMのゲフィチニブ添加によりEGFRのリン酸化は抑制され，ゲフィチニブはMRT細胞株に存在するEGFRのリン酸化を抑制することが確認された（POT）（図3）．

続いて，ゲフィチニブがMRT細胞株の増殖を抑制するかどうか検討した．まず，*in vitro*での細胞増殖は，培養細胞にゲフィチニブを添加し，24時間ごとに細胞数を計測した．MRT細胞株にゲフィチニブを0.1～100 μMで添加すると，48時間後から濃度依存性に増殖が抑制され，IC_{50}値はAN株が10 μMでNS株が12 μMであった．また，MRT細胞に20 μMのゲフィチニブを添加した場合，細胞は小球形に変形し浮

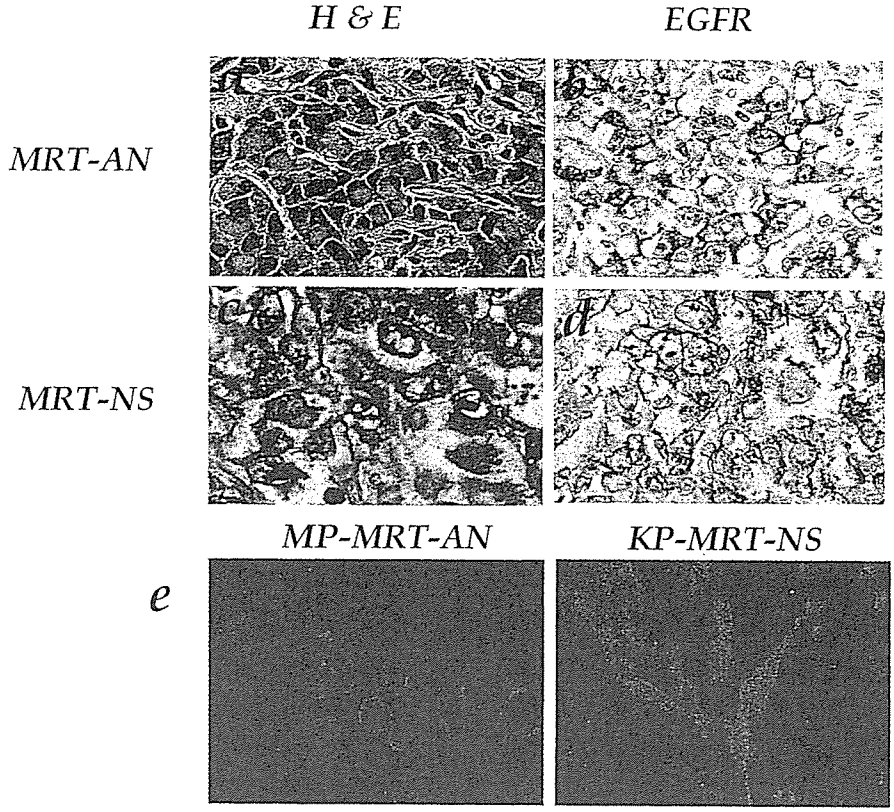

図2　MRT腫瘍組織と細胞株におけるEGFRの発現
a～d: 腫瘍組織MRT-ANとMRT-NSのH&E染色とEGFRの免疫染色．両腫瘍組織ともに細胞膜にEGFRの発現が認められた（×400）．e: 細胞株におけるEGFRの発現．間接蛍光抗体法によって，細胞膜にEGFRの発現が認められた．

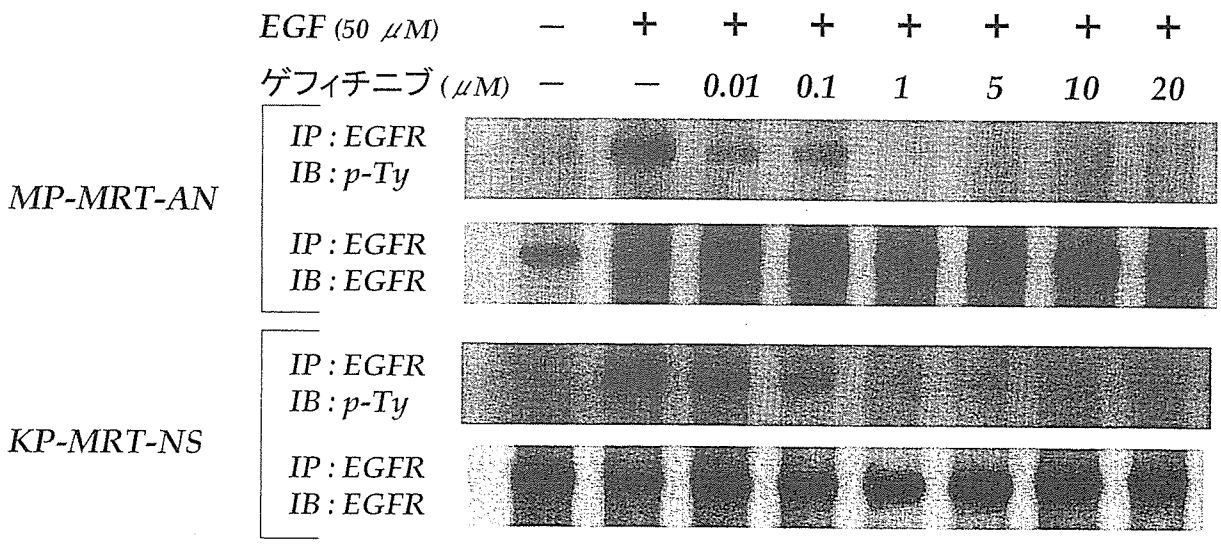

図3　ゲフィチニブによるEGFRのリン酸化の抑制
無血清ではEGFRのリン酸化は認められなかったが，EGFを添加するとEGFRはリン酸化された．しかし，ゲフィチニブを添加すると濃度依存的にEGFRのリン酸化が抑制された．

遊した．TUNEL法を用いてフローサイトメーターでアポトーシスを検討したところ，ゲフィチニブ20 μM投与群では10 μM投与群に比較し，有意に強くアポトーシスを誘導した(図4)．

さらに，*in vivo*での抗腫瘍効果を，MRT細胞株を背部皮下に移植したヌードマウスにゲフィチニブを経口投与し評価した．移植後7日目より，ゲフィチニブ経口投与群（150 mg/kg/日）とコントロール群とに分け，それぞれ週5日で計4週間投与し，腫瘍径で評価した．ゲフィチニブ投与により，AN株移植群とNS株移植群ともに，腫瘍の増殖は抑制された（図5）．

以上のように，EGFRを発現するMRT細胞に対して，ゲフィチニブはEGFRのリン酸化を抑制し，*in vitro*と*in vivo*ともに抗腫瘍効果を示した（POP）．すなわち，ゲフィチニブがMRTの患者の予後改善のために有用な分子標的治療薬の候補となりうる可能性が示されたものと考える．今後は，EGFRの発現と予後との関係や，発現するEGFRにおける遺伝子異常の検索（遺伝子変異や遺伝子のコピー数増加）などの検索が必要となろう．

ゲフィチニブの小児に対する使用状況と課題

アメリカのThe Childre's Oncology Group（COG）では2002年6月から2004年3月までの間に，再発し難治性となった固形腫瘍の小児の患者を対象に，ゲフィチニブの第Ⅰ相試験を行なった[14]．対象者は25人で年齢は中央値が14.8歳（1.8歳から21.2歳），神経芽腫，Wilms腫瘍，滑膜肉腫，Ewing肉腫などの腫瘍が含まれていた．投与量は150 mg/m²/日から300，400，500 mg/m²/日で，有害事象は米国国立癌研究所（NCI）の共通毒性基準（CTC）に基づいてgrade 4の肝機能障害が400 mg/m²/日で1例，500 mg/m²/日で

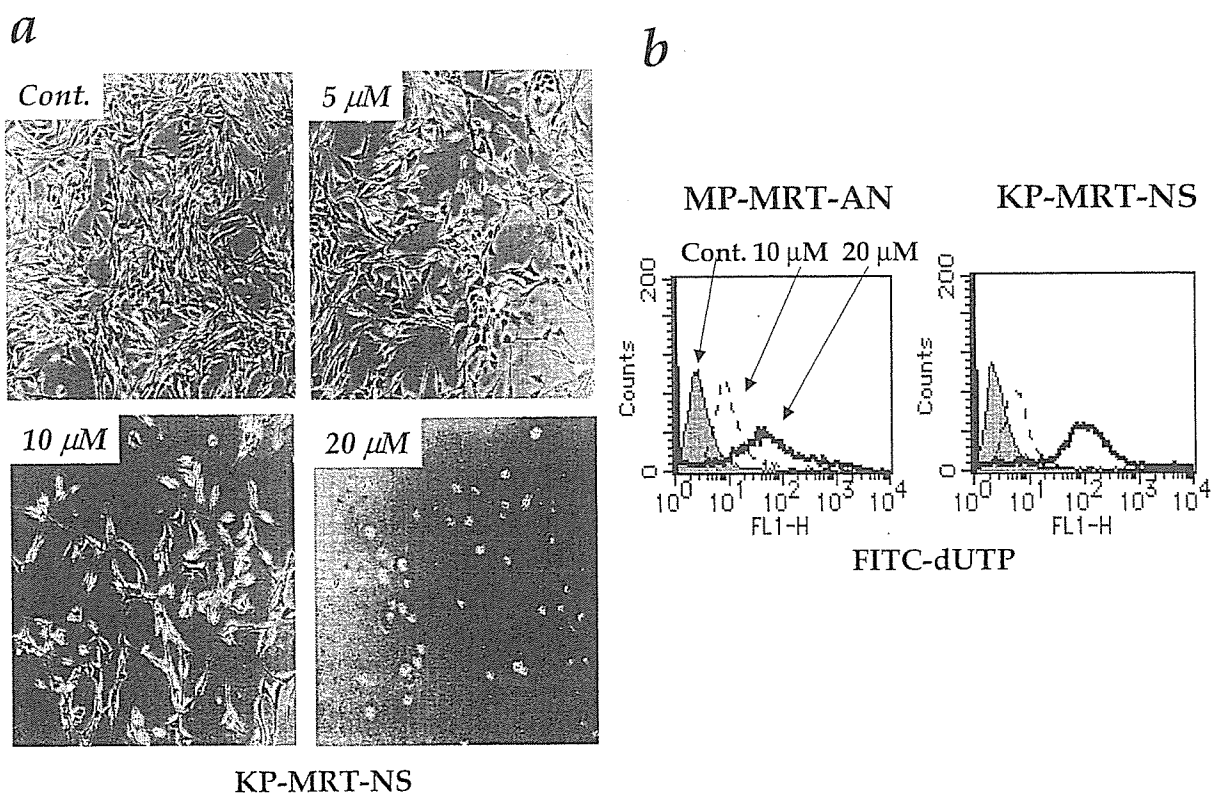

図4 *In virto*でのゲフィチニブのアポトーシス誘導効果

a: 血清培養液下で培養した細胞株にゲフィチニブを添加した後，96時間後の細胞株．ゲフィチニブは濃度依存的に増殖を抑制し，20 μMでは細胞が小球形に変形し浮遊する．図はKP-MRT-NS細胞株．

b: TUNEL法を用いフローサイトメーターでアポトーシスを検討した．ゲフィチニブ20 μM投与群は10 μM投与群よりアポトーシスを強く誘導した．

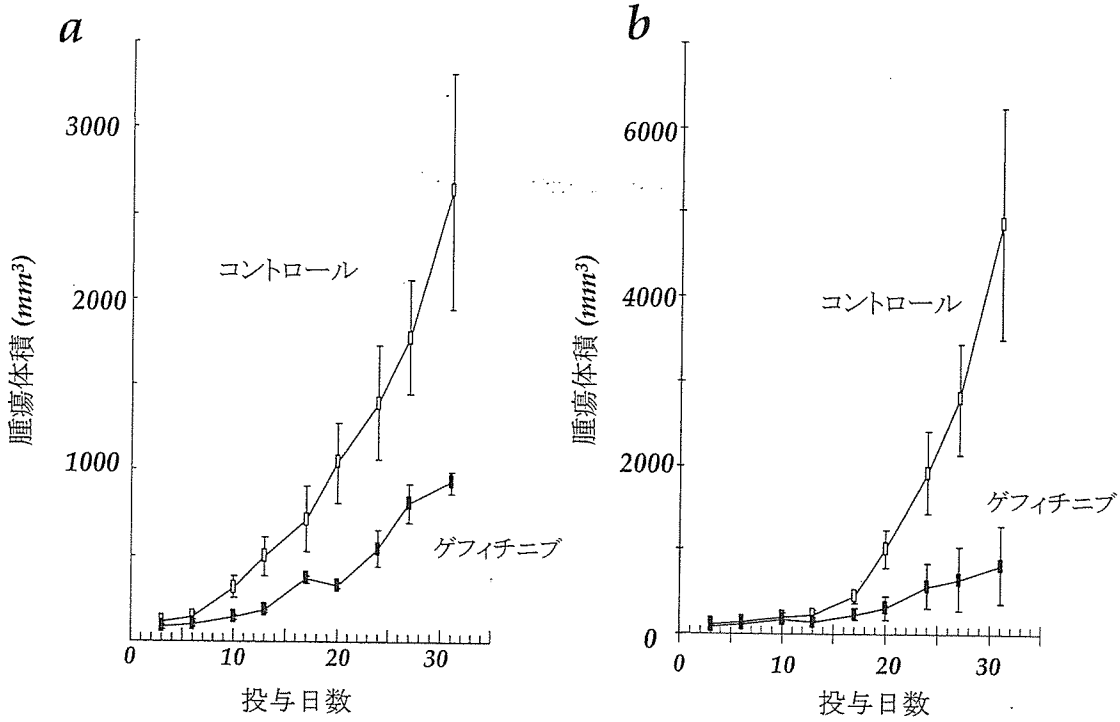

図5 *In vivo* における増殖抑制効果.

a: MP-MRT-AN 細胞株. ゲフィチニブ投与群では, 有意に腫瘍の増殖が抑制された（値は平均値±SE, $p=0.039$）.

b: KP-MRT-NS 細胞株. 同様に腫瘍の増殖が抑制された（値は平均値±SE, $p=0.048$）.

grade 3 と 4 の皮膚障害を 2 例認めた. しかし, 肺障害の観察例はなかった. この研究から, 最大耐用量は 400 mg/m²/日とされた. 投与量 400 mg/m²/日での最高血中濃度は平均 2.3 時間後で 2.2 μg/ml（1.2 μg/ml から 3.6 μg/ml まで）であった. 効果に関しては 150 mg/m²/日投与群の Ewing 肉腫の 1 例で有効性が認められた. すなわち, 小児でも耐用性が確認された.

この臨床試験ではゲフィチニブの血中濃度は平均で 2.2 μg/ml であったが, これは 4.92 μM に相当する. 我々の実験では IC_{50} 値は約 10 μM であったため, MRT に対する臨床効果に疑問も生じる. しかし, ゲフィチニブの場合, 血中濃度と腫瘍内の濃度を比較した場合, 腫瘍内濃度は約 42 倍高いと報告されている[15]. また, 平均年齢が 14.8 歳と比較的高い年齢層が対象であった. また, ゲフィチニブの主要な代謝酵素である CYP3A4 の発現は乳幼児で低いとされている[16]. こうした観点からは, 乳幼児で血中濃度, 腫瘍内濃度の十分な確保が期待できるものと考えられる.

しかし, 対象疾患が乳幼児の場合, ゲフィチニブの投与が有効であった場合, 投与期間が成人以上に長期間となる可能性がある. この場合, 耐性化が問題となってくるかもしれない. 実際, CML では, 二次的な遺伝子変異による, メシル酸イマチニブの耐性化が観察されているが, ゲフィチニブにおいても同様の耐性化が問題となってきている. 最近, ゲフィチニブ耐性化獲得と *EGFR* 遺伝子の T790M の二次的な変異の関与が報告されている[17]. 同様の変異を胚細胞系列に伴う家系の報告もあることから今後, 重要な問題となると考えられる.

また, 横紋筋肉腫, 神経芽腫や骨肉腫の細胞株を移植したマウスにゲフィチニブと CPT-11 を併用した場合, 単独よりも抗腫瘍効果が高くなるという報告がある[18]. しかも, EGFR の発現していない移植腫瘍でもその効果は見られることから, ゲフィチニブには, CPT-11 を細胞外へ排泄する ABCG2 を阻害し, CPT-11 の代謝活

性物質であるSN-38の効果を増強する作用があることが推察される．このように，ゲフィチニブはEGFR以外の重要な標的分子に作用している可能性が示唆される．プロテオミクスを用いてゲフィチニブの標的分子を20個以上も特定したという報告もある[19]．その中には，Src family kinaseやMETも含まれており，他の小児固形腫瘍にも応用が広がっていく可能性も考えられる．

最近，がん遺伝子の1つである*N-Ras*遺伝子の発現が，腫瘍組織によっては，腫瘍の進展に抑制的に働くという研究成果が発表された[20]．この事実は，がん遺伝子でも腫瘍の種類によっては，有効な分子標的にならない可能性を示唆している．したがって，新しい知見に基づいた治療効果の予測などを十分に行い，倫理性と科学性の保証された基礎研究と臨床試験が重要となる．

おわりに

小児悪性固形腫瘍における分子標的療法の現状と今後の可能性について，特にゲフィチニブ(gefitinib，イレッサ®)を中心に概説した．分子生物学の進歩は，今後も小児がんの悪性化の分子病態を解明するとともに，治療に有用と考えられる標的分子を明らかにするであろう．種々の難治性小児腫瘍に対する分子標的療法が確立し，それらの治療成績の向上のみならず，個々の患児の腫瘍病態にあわせたテーラーメイド治療の開発に大きく貢献することが期待される．ゲフィチニブは小児固形腫瘍の治療で，大きな可能性を持つ薬剤であると考えられる．そのためには，腫瘍別に標的分子を明確にし，基礎研究の成果を臨床へと繋ぐ，トランスレーショナルリサーチの基盤整備が必要である．最後に，小児固形腫瘍の後遺症を残さない治療を目指す上で，分子標的療法は，重要で期待される治療法であることを強調してこの稿を終えたい．

文献

1) Baselga J. Why the epidermal growth factor receptor? The rationale for cancer therapy. Oncologist 2002; 7: 2-9.

2) Bazeley LA, Gullick WJ. The epidermal growth factor receptor family.

3) Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, Nishiwaki Y, Vansteenkiste J, Kudoh S, Richin D, Eek R, Horai T, Noda K, Takata I, Smit E, Averbuch S, Macleod A, Feyereislova A, Dong RP, Baselga J. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung tumor. J Clin Oncol 2003; 21: 2237-2246.

4) Lynch T, Bell DW, sordella R, Gurubhagavtula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Setteleman J, Haber DA. Activation mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell-lung cancer to gefitinib. N Engl J Med 2004; 350: 2129-2139.

5) Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, Haney J, Witta S, Danenberg K, Dommenichini I, Ludovini V, Magrini E, Gregorc V, Doglioni C, Sidoni A, Tonato M, Franklin WA, Crino L, Bunn PA, Varella-Garcia M.: Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 2005; 97: 643-655.

6) Cappuzzo F, Magrini E, Ceresoli GL, Bartolini S, Rossi E, Ludovini V, Gregorc V, Ligorio C, Cancellieri A, Domiani S, Spreafico A, Paties CT, Lombardo L, Calandri C, Bellezza G, Hirsch FR, Tonato M, Crino L. Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. J Natl Cancer Inst 2004; 96: 1133-1141.

7) Bell DW, Lynch TJ, Haserlat SM, Harris PL, Okimoto RA, Brannigan BW, Sgroi DC, Muir B, Riemenschneider MJ, Iacona RB, Krebs AD, Johnson DH, Giaccone G, Herbst RS, Manegold C, Fukuoka M, Kris MG, Baselga J, Ochs JS, Haber DA. Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: Molecular analysis

of the IDEAL/INTACT gefitinib trials. J Clin Oncol 2005; 23: 8081-8092.
8) Bredel M, Pollack IF, Hamilton RL, James D. Epidermal growth factor receptor expression and gene amplification in high-grade non-brainstem gliomas of childfood. Clin Cancer Res 1999; 5: 1786-1792.
9) Sugimoto T, Kuroda H, Horii Y, Moritake H, Tanaka T, Hattori S. Signal transduction pathways through TRK-A and TRK-B receptors in human neuroblastoma. Jpn J Cancer Res 2001; 92: 152-160.
10) Ghanem MA, Van Der kwast TH, Den Hollander JC, Sudaryo MK, Mathoera RB, Van den Heuvel MM, Noordzij m, Nijiman RJM, van Steenbrugge GJ. Expression and prognostic value of epidermal growth factor receptor, transforming growth factor-α, and c-erbB-2 in nephroblastoma. Cancer 2001; 92: 3120-3129.
11) Gilbertson RJ, Perry RH, Kelly PJ, Pearson AD, Lunec J. Prognostic significance of HER2 and HER4 coexpression in childhood medulloblastoma. Cancer Res 1997; 57: 3272-3280.
12) Kuwahara Y, Hosoi H, Osone S, Kita M, Iehara T, Kuroda H, Sugimoto T. Antitumor activity of gefitinib in malignant rhabdoid tumor cells in vitro and in vivo. Clin Cancer Res 2004; 10: 5940-5948.
13) Tomlinson GE, Breslow NE, Dome J, Guthrie KA, Norkool P, Li S, Thomas PRM, Perlman E, Beckwith JB, D'Angio GJ, Green DM. Rhabdoid tomor of the kidney in the National Wilms' tumor study: Age at diagnosis as a prognostic factor. J Clin Oncol 2005; 23: 7641-7645.
14) Daw NC, Furman WL, Stewart CF, Iacono LC, Kralio M, Berstein ML, Dancey JE, Speights RA, Blaney SM, Croop JM, Reaman GH. Phase I and Pharmacokinetic study of gefitinib in childen with refractory solid tumors: A children's oncology group study. J Clin Oncol 2005; 23: 6172-6180.
15) Mckillop D, Partridge EA, Kemp JV, Spence MP, Kendrew J, Barnett S, Wood PG, Giles PB, Patterson AB, Bichat F, Guilbaud N, Stephens TC. Tumor penetration of gefitinib (Iressa), an epidermal growth factor receptor tyrosine kinase inhibitor. Mol Cancer Res 2005; 4: 641-649.
16) Johnson TN, Tanner MS, Taylor CJ, Tucker GT. Enterocytic CYP3A4 in a paediatric population: developmental change and the effect of coeliac disease and cystic fibrosis. Br J Clin Pharmacol; 51: 451-460.
17) Kobayashi S, Boggon T, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005; 352: 786-832.
18) Stewart CF, Leggas M, Schuetz JD, Panetta JC, Cheshire PJ, Peteson J, Daw N, Jenkins III JJ, Gilbertson RJ, Germain GS, Hawood FC, Houghton PJ. Gefitinib enhances the antitumor activity and oral bioavailability of irinotecan in mice. Cancer Res 2004; 64: 7491-7499.
19) Brehmer D, Greff Z, Godl K, Blencke S, Kurtenbach A, Weber M, Muller S, Klebl B, Cotton M, Keri G, Wissing J, Daub H. Cellular targets of gefitinib. Cancer Res 2005; 65: 379-382.
20) Takahashi C, Contreras B, Iwanaga T, Takegami Y, Bakker A, Bronson RT, Noda M, Loda M, Hunt JL, Ewen ME. Nras loss induces metastatic conversion of Rb1-deficient neuroendocrine thyroid tumor. Nature Genetics 2006; 38: 118-123.

Journal of Pediatric Surgery (2006) 41, 560–566

ELSEVIER

Journal of
Pediatric
Surgery

www.elsevier.com/locate/jpedsurg

Biological diagnosis for neuroblastoma using the combination of highly sensitive analysis of prognostic factors

Tatsuro Tajiri*, Shinji Tanaka, Mayumi Higashi, Yoshiaki Kinoshita, Yukiko Takahashi, Kyosuke Tatsuta, Sachiyo Suita

Department of Pediatric Surgery, Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan

Index words:
Neuroblastoma;
MYCN;
Survivin;
BIN1;
Quantitative PCR

Abstract

Background/Purpose: 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. This study aims to assess the correlation between the combination of prognostic factors and the biologic findings of neuroblastoma using a highly sensitive analysis of prognostic factors.

Methods: In 44 neuroblastoma primary samples, we determined the gene dosages of *MYCN* and *Survivin* (as the target of 17q gain) and the expression levels of *MYCN*, *Survivin*, and *BIN1* using highly sensitive analysis (the quantitative polymerase chain reaction method); furthermore, we assessed the correlation between the combination of their prognostic factors and the biology of neuroblastoma.

Results: The gene dosage of *MYCN* or *Survivin* was significantly associated with all known prognostic factors. The expression level of *MYCN* or *Survivin* was not significantly associated with any prognostic factors, whereas the expression level of *BIN1* was significantly associated with 5 of 6 prognostic factors. Regarding the combination of *MYCN* amplification and 17q gain (the gene dosage of *Survivin*), and the low expression of *BIN1*, the rates of advanced stages (stage III or IV) were 100% for the cases with 3 factors, 63% for the cases with 2 factors, 42% for the cases with 1 factor, and 0% for the cases with null factor. Furthermore, the survival rates were 20% for the cases with 3 factors, 50% for the cases with 2 factors, 100% for the cases with 1 factor, and 100% for the cases with null factor.

Conclusion: The combination of gene dosages of *MYCN* and *Survivin* and the expression level of *BIN1* using the quantitative polymerase chain reaction method was significantly correlated with the clinical stage and the patients' outcome. This combination of biologic factors may enhance the accuracy to the conventional criteria, but this would have to be shown in a much larger study that is adequately powered to detect such an advantage.

Neuroblastoma is the most common solid tumor in children, and its development is still unclear [1]. The prognosis in neuroblastoma tends to vary greatly, and many studies have demonstrated both clinical and biologic

* Corresponding author. Tel.: +81 92 642 5573; fax: + 81 92 642 5580.
E-mail address: taji@pedsurg.med.kyusyu-u.ac.jp (T. Tajiri).

0022-3468/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.jpedsurg.2005.11.067

factors to be correlated with the outcome [2]. To select the optimal treatment according to the degree of malignancy of neuroblastoma, it is essential to accurately and rapidly identify any genetic abnormality associated with the prognosis.

The amplification of the *MYCN* gene is the most unfavorable prognostic factor in neuroblastoma [3]. Approximately 20% to 30% of all patients presenting at advanced stages show an amplification of the *MYCN* gene, using the Southern blot method [4]. 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]. However, the clinical significance of *MYCN* expression in children with neuroblastoma remains controversial [6,7]. On the other hand, a gain of the chromosome 17q region has recently been implicated in close correlation with the aggressiveness of neuroblastoma, using either a comparative genomic hybridization study or the fluorescence in situ hybridization (FISH) method [8]. In particular, the gain of the q arm only has also been reported to play a role in an unfavorable outcome [9]. The region has been narrowed down to the 17q21-terminal, which is also considered to include the *Survivin* gene [10]. *Survivin* is a family member of an inhibitor of apoptosis proteins, and its expression is also cell cycle–regulated [11]. Recently, a high-level expression of *Survivin* in advanced stages of neuroblastoma has been shown, and it is thus considered to be one of the candidate genes for 17q gain [12]. We preliminary reported the quantitative polymerase chain reaction (PCR) method (TaqMan method) to be useful as a quick and accurate modality for evaluating for the status of *MYCN* amplification and the gene dosage of *Survivin* as the target of 17q gain in 25 neuroblastoma samples [13].

BIN1 (2q14) encodes multiple tissue-specific isoforms of an Myc-interacting adaptor protein that has features of a tumor suppressor, including the ability to inhibit Myc-mediated cell transformation and promote apoptosis [14]. We previously hypothesized that *BIN1* may function as a suppressor gene in neuroblastoma because *BIN1* is highly expressed in neural tissues and binds Myc within a region with 100% identity to *MYCN*, and reported data correlating reduced expression of *MYCN*-interacting *BIN1* isoforms with unfavorable features in primary neuroblastoma [15].

The quantitative TaqMan PCR System determines the initial copy number of the target gene by a kinetic analysis of the cycle-to-cycle change in the fluorescence signal as a result of the amplification of the template during PCR. Using this system, the detection of a loss of the 18q21 region in colon carcinoma tissue [16] and sex determination by defining the copy number of X chromosome have been reported. The advantage of quantitative PCR over Southern blotting, FISH, and comparative genomic hybridization is its speed (it takes about 4 hours from DNA extraction to the end of data analysis). Furthermore, this method is considered to have a high sensitivity [17].

In this study, we determined altogether the gene dosages of *MYCN* and *Survivin* (as the target of 17q gain) and the expression levels of *MYCN*, *Survivin*, and *BIN1* using highly sensitive analysis (the quantitative PCR method) in 44 neuroblastoma primary samples; furthermore, we assessed the correlation between the combination of their prognostic factors and the biology of neuroblastoma.

1. Materials and methods

1.1. Clinical data of patients and biologic data of neuroblastoma samples

Patients with neuroblastoma evaluated at the Department of Pediatric Surgery, Kyushu University, were diagnosed and staged according to the International Neuroblastoma Staging System [18]. Forty-four frozen tumor samples were obtained from untreated patients with neuroblastoma. The characteristics of the patients were shown to be as follows. The sex of the patients was 26 males and 18 females, and the age at diagnosis ranged from 19 days after birth to 11 years of age. Of the 44 cases, 15 patients were diagnosed at older than 1 year, whereas the remaining 29 were diagnosed at younger than 1 year. Twenty-four patients were identified by a neuroblastoma mass screening system. Of the 44 samples, 29 were tumors from patients who were stage I, II, or IVS, whereas 3 were stage III and 12 were stage IV. Thirty-six patients are still alive, of whom 3 cases are still under treatment, whereas 8 patients have died of the disease. The follow-up period after treatment ranged from 1 month to 12 years. In all 44 samples, the status of *MYCN* amplification was also previously determined by the Southern blotting method. In 36 of 44 cases with a single copy of *MYCN* identified by Southern blotting. DNA ploidy was previously examined using flow cytometry in 32 cases. Twenty-three cases were triploid, whereas 9 cases were diploid or tetraploid. Regarding the histologic findings, all 44 cases were classified based on the Shimada classification [19]. Thirty-two cases showed a favorable histology, whereas the remaining 12 cases showed an unfavorable histology. Regarding International Neuroblastoma Risk Group (INRG), all 44 cases were classified by age, stage (International Neuroblastoma Staging System), the status of *MYCN* amplification (Southern blot), DNA ploidy, and Shimada classification. Thirty cases showed a not high-risk group, whereas the remaining 14 cases showed a high-risk group.

1.2. DNA or RNA extraction and complementary DNA 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 was performed with a First-strand complementary DNA synthesis kit (Amersham Pharmacia, Uppsala, Sweden) using random hexanucleotide primers.

Table 1 The sequences of the PCR primers and TaqMan probes for quantitative PCR

Target gene	Forward primer	Reverse primer	TaqMan probe
MYCN	5′-CCC AGC GTG GTA GTC AAT GA-3′	5′-TTA ATG ACA AAG CCA TAA TCC ACA G-3′	5′-AGA ATG CGC ACA TGA TGC TAC ACG TTT CT -3′
Survivin	5′-GGG CTG CCA CGT CCA C-3′	5′-GTC GTC ATC TGG CTC CCA-3′	5′-TTC ATC CAC TGC CCC ACT GAG AAC GA-3′
p53	5′-GCC CTT ACT TGT CAT GGC GA-3′	5′-ATC CCA CAA CCC CTG CG-3′	5′-TGT CCA GCT TTG TGC CAG GAG CC-3′

1.3. Quantitative PCR (TaqMan)

As previously described, the *p53* gene was used as an internal control gene to obtain the gene dosage (*MYCN/p53*, *Survivin/p53*). The p53 gene is a tumor suppressor 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 [20]. The corrected gene dosage of the *MYCN* gene and *Survivin* gene was obtained based on the assumption that the mean gene dosage of 20 normal individual lymphocytes was 1.00. The mean ± 2SD of *MYCN* 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 mean ± 2SD of *Survivin* gene dosage of 20 normal individual lymphocytes was 1.00 ± 0.40. In this study, we evaluated that the *Survivin* amplified cells apparently present in the samples with a corrected gene dosage (*Survivin/p53*) of more than 1.50. The primers and TaqMan probes for the *MYCN* gene, *Survivin* gene, and the *p53* gene were designed using the application-based primer design software Primer Express (Applied Biosystems, Foster City, Calif). The sequences of the PCR primers and TaqMan probes were shown in Table 1. Quantitative PCR was performed in a final volume 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, 1× TaqMan PCR master mix, and 10 to 50 ng DNA. Thermal cycling was started with a 2 minutes 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 1 neuroblastoma with 90 copies of *MYCN* by Southern blotting method was serially diluted to establish the calibration curve.

1.4. Quantitative reverse transcriptase PCR (TaqMan)

The primers and TaqMan probes were designed to be located on exons 2 to 3 for *MYCN* messenger RNA (mRNA), exons 2 to 3 for *Survivin* mRNA, and on exons 9 to 11 for *BIN1* mRNA, hereby avoiding the amplification contaminating genomic DNA. *GAPDH* was used as an internal control gene to analyze the *MYCN* gene expression (*MYCN/GAPDH*). The sequences of the PCR primers and TaqMan probe were shown in Table 2. Polymerase chain reaction 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 quantitative reverse transcriptase polymerase chain reaction (RT-PCR) system was performed in the same manner as that for the quantitative PCR.

1.5. Statistical analysis

Fisher's Exact test was used to test the association between *MYCN* amplification (*MYCN/p53*, ≥2.0) or 17q gain (*Survivin/p53*, ≥1.50) and other prognostic factors. The expression levels of *MYCN* (*MYCN/p53*), *Survivin* (*Survivin/GAPDH*), and *BIN1* (*BIN1/GAPDH*) in the subgroups were represented by percentile (50%). A comparison of the gene dosage and expression in relation to clinical and genetic parameters was made using Mann-Whitney *U* test. Kruskal-Wallis exact test was used to test the association between the clinical stage or the patients' outcome and the combination of 3 prognostic factors.

Table 2 The sequences of the PCR primers and TaqMan probes for quantitative RT-PCR

Target gene	Forward primer	Reverse primer	TaqMan probe
MYCN	5′-GAC CAC AAG GCC CTC AGT ACC-3′	5′-TGA CCA CGT CGA TTT CTT CCT-3′	5′-CCG GAG AGG ACA CCC TGA GCG A-3′
Survivin	5′-GAC GAC CCC ATA GAG GAA CAT AA-3′	5′-GGG TTA ATT CTT CAA ACT GCT TCT TG-3′	5′-CGT CCG GTT GCG CTT TCC TTT CT-3′
BIN1	5′-AAG GCC CAG CCC AGT GAC-3′	5′-GAG CCA TCT GGA GGC GAA G-3′	5′-CGC GCC TGC AAA AGG GAA CAA GA-3′

2. Results

2.1. The gene dosages of MYCN and Survivin by the quantitative PCR method

Of the 36 samples with a single copy of *MYCN* based on the Southern blotting method, 33 samples showed the corrected gene dosage (*MYCN/p53*) to be less than 2.0, whereas the remaining 3 samples with more than 2.0 had tumors from patients with an advanced stage of disease (stages III and IV). Of the 3 samples with a dosage of more than 2.0, 2 cases died of the disease. In 8 cases with 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. In most of these cases, the analytic value based on the quantitative PCR was shown to be a higher than based on a Southern blotting analysis. The relationship between the *MYCN* gene dosage and the known prognostic factors (age, clinical stage, Shimada classification, INRG) is shown in Table 3. The group of cases with a gene dosage of more than 2.0 were strongly associated with an age of older than 1 year at diagnosis ($P < .001$), advanced stage ($P < .001$), a Shimada unfavorable histology ($P < .001$), and a high-risk group ($P < .001$), which are all unfavorable factors.

The corrected *Survivin* gene dosages ranged from 0.55 to 4.00. Ten cases showed that the *Survivin* gene dosages were more than 1.50-fold, and 6 of 10 cases were dead of disease. On the other hand, 32 of 34 cases with the *Survivin* gene dosages of less than 1.50-fold were free of disease. The relationship between the *Survivin* gene dosage and the known prognostic factors (age, clinical stage, Shimada classification, INRG) is shown in Table 3. The group of cases with a gene dosage of more than 2.0 was strongly associated with an age of older than 1 year at diagnosis ($P < .001$), advanced stage ($P < .001$), a Shimada unfavorable histology ($P < .001$), and a high-risk group ($P < .001$), which are all unfavorable factors.

Furthermore, we analyzed 20 samples that are clinically detected but not detected through mass screening. The relationship between the gene dosages *MYCN* or *Survivin* and the known prognostic factors was with the same trends compared with the results for all 44 samples. The gene dosage of *MYCN* was significantly associated with all 4 factors ($P < .01$), and the gene dosage of *Survivin* was significantly associated with 2 factors (age and Shimada, $P < .05$).

2.2. The expression level of MYCN, Survivin, and BIN1 by the quantitative RT-PCR method

The relationship between the *MYCN* gene, *Survivin* gene, or *BIN1* gene expression level and prognostic factors is shown in Table 4.

The level of *MYCN* expression in cases with *MYCN* amplification (*MYCN/p53*, ≥2.0) had a trend toward higher than that of cases with no *MYCN* amplification (*MYCN/p53*, <2.0); however, this finding was not statistically significant ($P = .15$). Furthermore, the expression level of *MYCN* was not significantly associated with any other prognostic factor (age, clinical stage, Shimada classification, the gene dosage of *MYCN* and *Survivin*, and INRG).

The level of *Survivin* expression was not significantly associated with the gene dosage of *Survivin*. In addition, the expression level of *Survivin* was not significantly associated with any other prognostic factor (age, clinical stage, Shimada classification, the gene dosage of *MYCN* and *Survivin*, and INRG).

The expression level of *BIN1* was significantly associated with 5 of 6 prognostic factors. Regarding 5 prognostic factors except the factor of age, the level of *BIN1* expression in neuroblastoma with the unfavorable factor was significantly lower than that in neuroblastoma with the favorable factor.

In addition, we analyzed 20 samples that are clinically detected but not detected through mass screening. The

Table 3 Gene dosage of *MYCN* and *Survivin* in relation to clinical and biologic prognostic factors

Category	n	*MYCN/p53*		*P*	*Survivin/p53*		*P*
		<2.0	≥2.0		<1.5	≥1.5	
Age (y)							
<1	29	27 (93.1)	2 (6.9)	<.01	28 (96.6)	1 (3.4)	<.01
≥1	15	6 (40.0)	9 (60.0)		6 (40.0)	9 (60.0)	
Stage							
Stage I, II, IVS	29	28 (96.6)	1 (3.4)	<.01	27 (93.1)	2 (6.9)	<.01
Stage III, IV	15	5 (33.3)	10 (66.7)		7 (46.7)	8 (53.3)	
Shimada							
Favorable	32	30 (93.8)	2 (6.2)	<.01	30 (93.8)	2 (6.2)	<.01
Unfavorable	12	3 (25.0)	9 (75.0)		4 (33.3)	8 (66.7)	
INRG							
Not high risk	30	29 (96.7)	1 (3.3)	<.01	28 (93.3)	2 (6.7)	<.01
High risk	14	4 (28.6)	10 (71.4)		6 (42.9)	8 (57.1)	

Values are presented as n (%). *P* value was determined by Fisher's Exact test.

Table 4 Expression of *MYCN*, *Survivin*, and *BIN1* in relation to clinical and biologic prognostic factors

Category	n	*MYCN*/GAPDH 50 percentile	*P*	*Survivin*/*GAPDH* 50 percentile	*P*	*BIN1*/*GAPDH* 50 percentile	*P*
Age (y)							
<1	29	0.22	.78	0.30	.79	1.17	.08
≥1	15	0.25		0.32		0.41	
Stage							
Stage I, II, IVS	29	0.15	.36	0.28	.42	1.36	<.01
Stage III, IV	15	0.52		0.5		0.41	
Shimada							
Favorable	32	0.19	.51	0.29	.43	1.21	<.01
Unfavorable	12	0.44		0.41		0.21	
Survivin/p53							
<1.5	34	0.31	.20	0.40	.94	1.15	<.05
≥1.5	10	0.08		0.27		0.38	
MYCN/p53							
<2.0	33	0.15	.15	0.34	.79	1.50	<.01
≥2.0	11	0.52		0.25		0.07	
INRG							
Not high risk	30	0.15	.33	0.29	.35	1.31	<.01
High risk	14	0.49		0.41		0.21	

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

relationship between the expression level of *MYCN*, *Survivin*, and *BIN1*, and the known prognostic factors was with the same trends compared with the results for all 44 samples. The expression level of *BIN1* was significantly associated with 4 factors (clinical stage, Shimada classification, the gene dosage of *MYCN*, and INRG; $P < .05$).

2.3. Evaluation of biology for neuroblastomas using the combination of 3 prognostic factors

In the highly sensitive analysis of prognostic factors in this study, the gene dosage of *MYCN*, the gene dosage of *Survivin*, and the level of *BIN1* expression were significant prognostic factors. The relationship between these 3 unfavorable prognostic factors (*MYCN* amplification—*MYCN/p53*, ≥2.0; 17q gain—*Survivin/p53*, ≥1.50; the low expression of *BIN1*—*BIN1/GAPDH*, <1.0) and clinical behavior (clinical stage and outcome) is shown in Fig. 1. Regarding the combination of *MYCN* amplification and 17q gain, and the low expression of *BIN1*, the rates of advanced stages (stages III and IV) were 100% for the cases with 3 factors, 63% for the cases with 2 factors, 42% for the cases with 1 factors, and 0% for the cases with null factor ($P < .001$, trend test using Kruskal-Wallis exact test). Furthermore, the survival rates were 20% for the

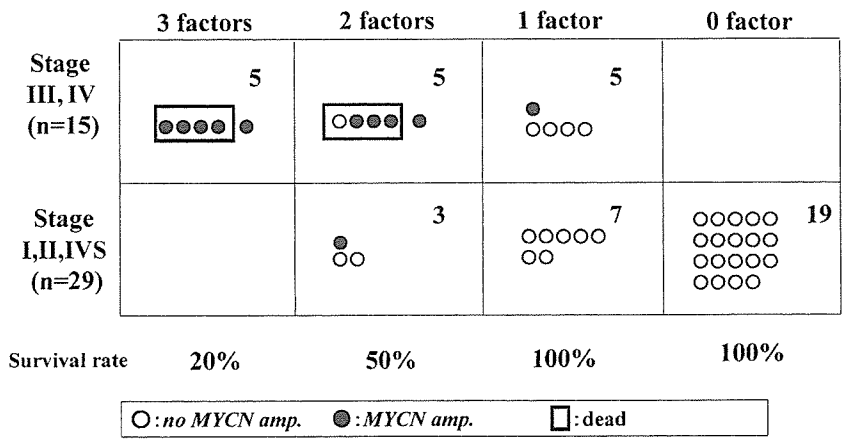

Fig. 1 The correlation of 3 unfavorable factors (*MYCN* amplification—*MYCN/p53*, ≥2.0; 17q gain—*Survivin/p53*, ≥1.50; the low expression of *BIN1*—*BIN1/GAPDH*, <1.0) and the clinical behavior in neuroblastomas. The rates of advanced stages (stages III and IV) were 100% for the cases with 3 factors, 63% (5/8) for the cases with 2 factors, 42% (5/12) for the cases with 1 factors, and 0% (0/19) for the cases with null factor ($P < .001$, trend test using Kruskal-Wallis exact test). The survival rates were 20% for the cases with 3 factors, 50% for the cases with 2 factors, 100% for the cases with 1 factor, and 100% for the cases with null factor ($P < .001$, trend test using Kruskal-Wallis exact test).

cases with 3 factors, 50% for the cases with 2 factors, 100% for the cases with 1 factor, and 100% for the cases with null factor ($P < .001$, trend test using Kruskal-Wallis exact test).

3. Discussion

Neuroblastomas have a variety of genetic variables that might predict the clinical behavior [2]. To select the optimal treatment according to the degree of malignancy of neuroblastoma, it is essential to accurately and rapidly identify any genetic heterogeneity associated with the prognosis. Generally, the gene dosage was analyzed by Southern blot method or FISH, whereas the expression level of gene was assessed by Northern blot method or semiquantitative PCR method. We previously reported that the quantitative PCR method may be considered to be the most effective methods for quickly and accurately evaluating any aberration in the gene dosages associated with the patients' outcomes [13,21]. In this study, we determined altogether the gene dosages of *MYCN* and *Survivin* (as the target of 17q gain) and the expression levels of *MYCN*, *Survivin*, and *BIN1* using highly sensitive analysis (the quantitative PCR method) in 44 neuroblastoma primary samples.

Regarding the *MYCN* gene, the amplification of the *MYCN* gene is strongly associated with rapid tumor progression [3,4]; however, the clinical significance of *MYCN* expression in children with neuroblastoma remains controversial [6,7]. In the present study, the gene dosage of *MYCN* was significantly associated with all prognostic factors, whereas the expression level was not significantly associated with any prognostic factor. Furthermore, the significant association between the gene dosage and the expression level was not observed. These findings are suggesting that the only gene dosage of *MYCN* does not always contribute to the level of *MYCN* expression in neuroblastoma, and the expression level of *MYCN* does not seem to be an independently significant prognostic factor in this highly sensitive analysis.

Regarding the *Survivin* gene, we assumed the *Survivin* gene could be one of the candidate genes for the 17q gain in neuroblastoma. In the present study, the gene dosage of *Survivin* was significantly associated with all prognostic factors, whereas the expression level was not significantly associated with any prognostic factor. In addition, there was no correlation between the *Survivin* gene dosage and the expression level. These results are demonstrating that analysis of the gene dosage of *Survivin* is useful for evaluating the 17q gain; however, the *Survivin* was not the candidate gene for 17q gain in neuroblastoma.

It is unclear why gene dosage is more correlative with risk than expression level. The chromosomal gain or loss may be correlated with the genomic instability, which is associated with poor prognosis in adult cancers [22]. The genomic instability may generate *MYCN* amplification or 17q gain as one of chromosomal alteration in neuroblastoma.

Taken together, the gene dosage of *MYCN* (*MYCN* amplification), the gene dosage of *Survivin* (17q gain), and the level of *BIN1* expression were significant prognostic factors in the highly sensitive analysis using the quantitative PCR method. Furthermore, the combination of gene dosages of *MYCN* and *Survivin* and the expression level of *BIN1* using the quantitative PCR method was substantially correlated with the clinical stage and the patients' outcome. The current protocol for neuroblastoma in the world is mainly based on the age, clinical stage, and *MYCN* amplification. In the Study Group of Japan for Advanced Neuroblastoma, 2 chemotherapeutic regimens for advanced neuroblastoma have been designed based on the *MYCN* amplification status since 1991 [23]. However, the status of *MYCN* amplification does not necessarily predict the patients' outcome. This combination of biologic factors may enhance the accuracy to the conventional criteria (*MYCN*, Shimada classification), but this would have to be shown in a much larger study that is adequately powered to detect such an advantage.

References

[1] Crist WM, Kun LE. Common solid tumors of childhood. N Engl J Med 1991;324:461-71.

[2] Brodeur GM. Molecular basis for heterogeneity in human neuroblastoma. Eur J Cancer 1995;31:505-10.

[3] Brodeur GM, Maris JM, Yamashiro DJ, et al. Biology and genetics of human neuroblastomas. J Pediatr Hematol Oncol 1997;19:93-101.

[4] Brodeur GM, Seeger RC, Schwab M, et al. Amplification of *MYCN* in untreated human neuroblastomas correlates with advanced disease stage. Science 1984;224:1121-4.

[5] Lutz W, Stohr M, Schurmann J, et al. Conditional expression of N-*myc* in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. Oncogene 1996;13:803-12.

[6] Bordow SB, Norris MD, Haber PS, et al. Prognostic significance of *MYCN* oncogene expression in childhood neuroblastoma. J Clin Oncol 1998;16:3286-94.

[7] Seeger RC, Wada R, Brodeur GM, et al. Expression of N-*myc* by neuroblastomas with one or multiple copies of the oncogene. Prog Clin Biol Res 1998;271:41-9.

[8] Caron HN. Allelic loss of chromosome 1 and additional chromosome 17 material are both unfavorable prognostic markers in neuroblastoma. Med Pediatr Oncol 1995;24:215-21.

[9] Lastowska M, Cotterill S, Pearson ADJ, et al. Gain of chromosome arm 17q predicts unfavorable outcome in neuroblastoma patients. Eur J Cancer 1997;33:1627-33.

[10] Plantaz D, Mohapatra G, Matthay KK, et al. Gain of chromosome 17 is the most frequent abnormality detected in neuroblastoma by comparative genomic hybridization. Am J Pathol 1997;150:81-9.

[11] Adida C, Crotty PL, McGrath J, et al. Developmentally regulated expression of the novel cancer anti-apoptosis gene *Survivin* in human and mouse differentiation. Am J Pathol 1998;19:617-23.

[12] Islam A, Kageyama H, Takada N, et al. High expression of *Survivin*, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene 2000;19:617-23.

[13] Tajiri T, Tanaka S, Shono K, et al. Quick quantitative analysis of gene dosages associated with prognosis in neuroblastoma. Cancer Lett 2001;166:89-94.
[14] Hogarty MD, Liu X, Thompson PM, et al. *BIN1* inhibits colony formation and induces apoptosis in neuroblastoma cell lines with *MYCN* amplification. Med Pediatr Oncol 2000;35:559-62.
[15] Tajiri T, Tanaka S, Suita S, et al. Expression of a *MYCN*-interacting isoform of the tumor suppressor *BIN1* is reduced in neuroblastomas with unfavorable biological features. Clin Cancer Res 2003;9:3345-55.
[16] Laurendeau I, Bahuau M, Vodovar N, et al. TaqMan PCR–based gene dosage assay for predictive testing in individuals from a cancer family with INK 4 locus haploinsufficiency. Clin Chem 1999;45: 982-6.
[17] Kariyazono H, Ihara K, Ohno T, et al. Rapid detection of 22q11.2 deletion with quantitative real-time PCR. Mol Cell Probes 2001; 15:71-3.
[18] Brodeur GM, Pritchard J, Berthold F, et al. Revision of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 1993;11:1466-77.
[19] Shimada H, Chatten J, Newton WA. Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglio-neuroblastoma and an age-linked classification of neuroblastomas. J Natl Cancer Inst 1984;73:405-16.
[20] Kusafuka T, Fukuzawa M, Oue T, et al. Mutation analysis of p53 gene in childhood malignant solid tumors. J Pediatr Surg 1997;32:1175-80.
[21] Tanaka S, Tajiri T, Suita S, et al. Clinical significance of a highly sensitive analysis for gene dosage and the expression level of *MYCN* in neuroblastoma. J Pediatr Surg 2004;39:63-8.
[22] Diaz Jr LA. The current clinical value of genomic instability. Semin Cancer Biol 2005;15:67-71.
[23] Kaneko M, Nishihira H, Mugishima H, et al. Stratification of treatment of stage 4 neuroblastoma patients based on N-*myc* amplification status. Med Pediatr Oncol 1999;31:1-7.

Journal of Pediatric Surgery (2006) 41, 1501–1505

ELSEVIER

Journal of Pediatric Surgery

www.elsevier.com/locate/jpedsurg

Original articles

Clinical characteristics and outcome of Wilms tumors with a favorable histology in Japan: a report from the Study Group for Pediatric Solid Malignant Tumors in the Kyushu Area, Japan

Sachiyo Suita[a,*], Yoshiaki Kinoshita[a], Tatsuro Tajiri[a], Toshiro Hara[b], Masazumi Tsuneyoshi[c], Hiroyoshi Mizote[d], Hiroko Inada[e], Hideo Takamatsu[f], Yoshifumi Kawano[g], Yukihiro Inomata[h], Akira Nagasaki[i], Yasuharu Ono[j], Noritoshi Handa[k], Jun Okamura[l], Eiichi Ishii[m], Kiyoshi Kawakami[n], The committee for pediatric solid malignant tumors in the Kyushu area

[a]*Department of Pediatric Surgery, Kyushu University, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan*
[b]*Department of Pediatrics, Kyushu University, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan*
[c]*Department of Anatomic Pathology, Kyushu University, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan*
[d]*Department of Pediatric Surgery, Kurume University, Kurume 830-0011, Japan*
[e]*Department of Pediatrics, Kurume University, Kurume 830-0011, Japan*
[f]*Department of Pediatric Surgery, Kagoshima University, Kagoshima 890-8520, Japan*
[g]*Department of Pediatrics, Kagoshima University, Kagoshima 890-8520, Japan*
[h]*Department of Pediatric Surgery, Kumamoto University, Kumamoto 860-8556, Japan*
[i]*Department of Surgery, Fukuoka Children's Hospital, Chuo-ku, Fukuoka 810-0063, Japan*
[j]*Division of Pediatric Surgery, Department of Surgery, Nagasaki University, Nagasaki 852-8501, Japan*
[k]*Department of Pediatric Surgery, Oita Prefectural Hospital, Oita 870-8511, Japan*
[l]*Division of Pediatrics, National Kyushu Cancer Center, Minamita, Fukuoka 811-1395, Japan*
[m]*Department of Pediatrics, Saga University, Saga 849-8501, Japan*
[n]*Department of Pediatrics, Kagoshima City Hospital, Kagushima 892-8580, Japan*

Index words:
Wilms tumor;
Nephroblastoma;
Outcome
JWiTS

Abstract

Background/Purpose: Since 1996, the standard treatment of Wilms tumors in Japan has been based on the regimen of the Japanese Wilms Tumor Study. However, in Japan, there have been no reports about Wilms tumors that analyzed the clinical features and patient outcome in a large series until now. This study aims to assess the clinical characteristics of patients with Wilms tumor with a favorable histology from a retrospective standpoint in the Kyushu area in Japan and, furthermore, to analyze the historical changes of clinical features and outcome from the 1980s to the 1990s.

* Corresponding author. Pediatric Surgery Reproductive and Developmental Medicine Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 5568 fax: +81 92 642 5580.
E-mail address: suita@pedsurg.med.kyushu-u.ac.jp (S. Suita).

0022-3468/$ – see front matter
doi:10.1016/j.jpedsurg.2006.05.008

Methods: Between 1982 and 1996, 90 cases of Wilms tumors with a favorable histology were registered in the Kyushu area. Regarding the clinical feature and outcome, they were divided into 2 groups (group A, 1982-1989, n = 50; group B, 1990-1996, n = 40). The outcome was analyzed based on the 5-year overall survival rate.

Results: The clinical features (age, sex, initial symptom, location, stage) demonstrated no definite differences between group A and group B. Regarding the operation, the rate of an initial complete resection in the early stages was significantly higher in group B than in group A. All stage V cases in group B undewent a bilateral tumor biopsy instead of a radical nephrectomy as the initial operation. The 5-year overall survival rate throughout the whole period was 87.8%, whereas the rates were 84.0% for group A and 90.0% for group B (P = NS), respectively. Of particular note, the outcome of patients with stage I and stage V in group B substantially improved in comparison to that in group A. However, in advanced cases, no significant improvement in the outcome was noted.

Conclusions: This is the first report about the clinical features and outcome for Wilms tumors with a favorable histology in Japan from the 1980s to the 1990s. The present study suggested that in the early-stage cases, an initially complete resection followed by standard postoperative chemotherapy substantially improved the outcome of the patients in group B. In the stage V cases, the performance of renal salvage surgery may have positively contributed to the improvement in the outcome in group B. However, in the advanced stage cases, no definite improvement was noted. In the future, an improved efficacy of the treatments for Wilms tumors based on the standard protocol established by the Japanese Wilms Tumor Study in 1996 is expected in Japan.

Wilms tumor is the most common malignant neoplasm of the kidney observed in children. The multimodality treatment for such tumors, including surgery, chemotherapy, and radiotherapy, has undergone refinement in recent years. The National Wilms Tumor Study (NWTS) was established in 1969 as a central organization for the treatment of Wilms tumor in the United States and Canada. Standard protocols were established according to the clinical stage and histology. Some modifications have been made over the past 35 years, and NWTS-5 is continuing its activities up to the present. On the other hand, in Europe, the International Society of Pediatric Oncology (SIOP) was the central organization for the treatment of Wilms tumor. The NWTS recommends an initial radical operation, followed by adjuvant chemotherapy and radiotherapy, whereas the SIOP emphasizes the efficacy of prenephrectomy treatment.

In Japan, about 40 to 50 cases of Wilms tumor occur every year. The treatment regimen in Japan depended on the decision of each institution until the Japanese Wilms Tumor Study (JWiTS), which was based on the NWTS-4 regimen, was established in 1996. In Japan, there have so far been no reports on Wilms tumors that analyzed the clinical features and patients outcomes in such a large series until now. In the current study, we retrospectively assessed the clinical characteristics of patients with Wilms tumor with a favorable histology from the Kyushu area in Japan and, furthermore, analyzed the historical changes in the clinical features and outcome from the 1980s to 1990s.

1. Material and methods

From 1982 to 1996, 98 patients with Wilms tumor from the Kyushu Area of Japan were registered by the Committee for Pediatric Solid Malignant Tumors. Of the 98 patients, 90 had a favorable histology, whereas 8 had an unfavorable histology. In this study, the patients with a favorable histology were divided into 2 groups (group A, 1982-1989, n = 50; group B, 1990-1996, n = 40) to assess the historical changes of the clinical features and outcome in the patients with Wilms tumor. They were analyzed for the following clinical characteristics: age, sex, initial symptoms, location, stage, treatment, and prognosis. The staging was performed according to the classification system developed by the committee for malignant tumors of the Japanese Society of Pediatric Surgeons. The 5-year overall survival distributions were estimated using the method of Kaplan and Meier. The survival time was defined as the time from the initial diagnosis to death. The log-rank test was used to assess any statistical significance in the prognostic factors.

2. Results

2.1. Clinical features and treatments of the 90 cases of Wilms tumors

The mean age of the patients was 3.0 ± 3.1 years in group A and 2.6±1.9 years in group B. There was a higher percentage of boys in both groups. Regarding the initial symptoms, abdominal tumor was the most common symptom in both group A (74.0%) and group B (64.0%). The tumor location was more frequently observed on the left side both in group A (53.0%) and group B (65.6%). For the bilateral cases, 3 cases (7.5%) were observed in group A, and 6 cases (12.0%) were observed in group B. Regarding the clinical stage, 23 were stage I, 13 were stage II, 5 were stage III, 6 were stage IV, and 3 were stage V in

Table 1 Clinical features

	Total	Group A (n = 50)	Group B (n = 40)
Age (y)	2.8 ± 2.6	3.0 ± 3.1	2.6 ± 1.9
Sex			
Boy	53	29	24
Girl	37	21	16
Initial symptom (%)			
Abdominal tumor	63.0	74.0	64.0
Hematuria	27.9	24.0	25.0
Abdominal pain	13.5	12.0	17.5
Abdominal distension	11.7	10.0	22.5
Fever	11.7	18.0	20.0
Location			
Left	49	26	23
Right	32	21	11
Bilateral	9	3	6
Stage			
I	39	23	16
II	27	13	14
III	8	5	3
IV	7	6	1
V	9	3	6

group A. On the other hand, 16 were stage I, 14 were stage II, 3 were stage III, 1 was stage IV, and 6 were stage V in group B (Table 1).

An initial complete resection was possible for 77 cases throughout the entire study period. The rate of an initial complete resection for each stage was as follows (Table 2): group A—87.0% (stage I), 76.9% (stage II), 20.0% (stage III), 16.7% (stage IV), and 66.7% (stage V); group B—93.8% (stage I), 85.7% (stage II), 0.0% (stage III), 0.0% (stage IV), and 0.0% (stage V).

2.2. The association between clinical stage and patient outcome

The 5-year overall survival rate is demonstrated in the Table 3. For all cases, the 5-year overall survival rate throughout the entire study period was 87.8%, and the rates were 84.0% in group A and 90.0% in group B (P = NS). In the early stages, the 5-year overall survival rate was 83.3% and 100.0% for stage I ($P < .05$) (Fig. 1) and 100.0% and 100.0% for stage II (P = NS) for group A and group B,

Table 2 Initially complete resection of the primary tumor

	Total (n = 90)	Group A (n = 50)	Group B (n = 40)
Stage I	89.7	87.0	93.8
Stage II	81.5	76.9	85.7
Stage III	12.5	20.0	0.0
Stage IV	14.2	16.7	0.0
Stage V	22.2	66.7	0.0

Values are presented as percent.

Table 3 The 5-year overall survival rate

	Total (%)	Group A (%)	Group B (%)	P
All cases[a]				
Total	87.8	84.0	90.0	NS
Stage I and Stage II (early stages)[b]				
Stage I	92.3	86.9	100.0	<.05
Stage II	100.0	100.0	100.0	NS
Total	95.5	89.3	100.0	<.05
Stage III and Stage IV (advanced stages)[c]				
Stage III	75.0	80.0	66.7	NS
Stage IV	57.1	66.7	0.0	NS
Total	66.7	66.7	66.7	NS
Stage V[d]				
Total	77.8	33.3	100.0	<.05

NS indicates not significant.
[a] Total, n = 90; group A, n = 50; group B, n = 40.
[b] Total, n = 66; group A, n = 36; group B, n = 30.
[c] Total, n = 15; group A, n = 11; group B, n = 4.
[d] Total, n = 9; group A, n = 3; group B, n = 6.

respectively. Regarding all early-stage cases, a significant improvement in the outcome was seen in group B. In the advanced stage cases, the rates were 80.0% and 66.7% for stage III (P = NS) and 66.7% and 0.0% for stage IV (P = NS) for group A and group B, respectively. In addition, for the bilateral stage V cases, the survival rate was 33.3% and 100.0% ($P < .05$) for group A and group B, respectively (Fig. 2).

3. Discussion

NWTS-3 (1979-1986) demonstrated that the 4-year overall survival rate of Wilms tumors with a favorable histology were 96.5% for stage I, 92.2% for stage II, 86.9% for stage III, 73.0% for stage IV, and 76.0% for stage V [1,2]. On the other hand, SIOP-9 (1987-1991) reported 100% for stage I, 88% for stage II, 85% for stage III, and 83% for stage IV [3,4].

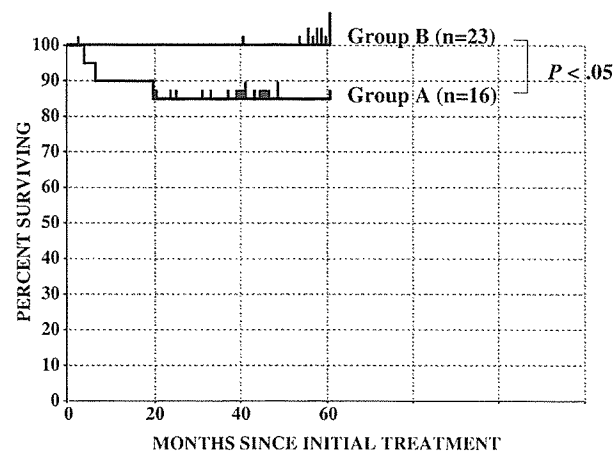

Fig. 1 The 5-year overall survival rate (stage I).

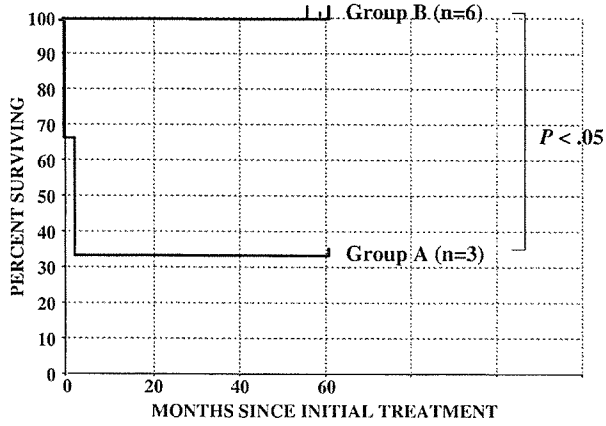

Fig. 2 The 5-year overall survival rate (stage II).

In the present study, the 5-year overall survival rate was 87.8% for all cases. The rates for each stage were as follows: stage I, 92.3%; stage II, 100.0%; stage III, 75.0%; stage IV, 57.1%; and stage V, 77.8%. Regarding the early stages and stage V, these results were never inferior to those of NWTS-3 and SIOP-9; however, in the advanced stage, the results are evidently inferior. The historical analyses also demonstrated that the improvement in the outcome has been noted in the early stages and stage V, whereas it was not noted in the advanced stages in group B.

In the early-stage cases, the rate of an initially complete resection in group B was higher than that in group A. The reason for this is considered to be the treatment based on the NWTS protocol, namely, an initial radical operation followed by standard postoperative chemotherapy. As a result, a significant improvement in the outcome has been noted in the early stage cases in group B.

Regarding the therapy for stage V cases, the risk of end-stage renal failure in bilateral cases exceeded 9% because of a 2-staged bilateral nephrectomy in NWTS reports [5]. A nephrectomy for unilateral tumors increases the risk of hypertension, proteinuria, glomerulosclerosis, and renal insufficiency. On the other hand, the average tumor volume reduction exceeds 50% with chemotherapy [6]. The initial operation should thus be minimized by performing a biopsy of the bilateral lesions whenever possible to avoid an invasive operation. After decreasing the size of the bilateral tumors, minimum invasive surgery should be performed [7-10]. In our series, 2 (66.7%) of 3 patients underwent a radical operation including a unilateral nephrectomy and a contralateral biopsy in group A, whereas all the cases in group B were fundamentally treated with a renal salvage "nephron-sparing surgery" operation. In our current study, the induction of such an operation contributed to the favorable outcome in bilateral Wilms tumors. In the future, the preservation of the renal parenchyma by performing bench surgery is expected to become widespread [11].

On the other hand, in the advanced-stage cases, including stage III and stage IV, no definite improvement in the outcome was noted in group B. The results were therefore evidently inferior to those of NWTS-3 or SIOP-9 during almost the exact same period.

Regarding chemotherapy, in the advanced stage cases, NWTS-3 and SIOP-9 demonstrated the effectiveness of a 3-drug treatment using doxorubicin added to a 2-drug regimen consisting of vincristine and dactinomycin [12,13]. In our series of stage III cases, although the number of patients was small, a 2-drug regimen (vincristine and dactinomycin) was used for all patients in group A. However, in group B, 2 of 3 patients were treated with a 3-drug regimen (vincristine, dactinomycin, and doxorubicin) as preoperative chemotherapy, and these 2 patients survived for over 5 years (data not shown). Although our findings are still inconclusive, the 3-drug chemotherapeutic regimen appears to be highly effective.

Regarding the use of radiotherapy in the advanced-stage cases, NWTS-3 and SIOP-9 emphasized the role of radiotherapy and also reported an excellent prognosis exceeding 80% for stage IV cases. In our series, all cases in both groups underwent postoperative radiotherapy followed by the NWTS regimen mainly (data not shown). However, the efficacy of the radiotherapy in our series was not evident from these data alone.

In Japan, the JWiTS regimen is fundamentally based on the same concept of NWTS, and an initially complete resection followed by postoperative standard chemotherapy and radiotherapy is recommended. In our series, the treatment of early-stage cases was almost always based on the NWTS protocol. However, in many advanced-stage cases, an initially complete resection was impossible, and some cases were thus forced to undergo preoperative chemotherapy based on the decision of each institution. Regarding bilateral cases, nephron-sparing surgery clearly improved the outcome.

In conclusion, the present study suggested that in the early-stage cases, an initially complete resection followed by the standard postoperative chemotherapy clearly improved the outcome for the patients in group B. In the stage V cases, the performance of renal salvage surgery contributed positively to the improved outcome in group B. However, in advanced cases, no definite improvement has been noted. In the future, an improved efficacy in the treatments for Wilms tumor based on the standard protocol by JWiTS is expected, especially for the advanced-stage cases in Japan.

References

[1] D'Angio GJ, Breslow N, Beckwith JB, et al. Treatment of Wilms tumor: results of the third National Wilms Tumor Study. Cancer 1989;64:349-60.

[2] Breslow NE, Ou SS, Beckwith JB, et al. Doxorubicin for favorable histology, stage II-III Wilms tumor: results from the National Wilms Tumor Studies. Cancer 2004;101:1072-80.

[3] Tournade F, Com-Hougue C, De Kraker J, et al. Optimal duration of preoperative chemotherapy in unilateral and nonmetastatic Wilms

tumor in children older than 6 months: results of the ninth International Society of Pediatric Oncology Wilms tumor trial and study. J Clin Oncol 2001;19:488-500.

[4] Graf N, Tournade MF, de Kraker J. The role of preoperative chemotherapy in the management of Wilms tumor. Urol Clin North Am 2000;27:443-54.

[5] Ritchey ML, Green DM, Thomas PRM, et al. Renal failure in Wilms tumor patients: a report from the National Wilms Tumor Study Group. Med Pediatr Oncol 1999;26:75-80.

[6] Lemerle J, de Kraker J, Tournade MF. The SIOP Wilms trials and studies. Med Pediatr Oncol 1993;21:553.

[7] Horwitz JR, Ritchey ML, Moksness J, et al. Renal salvage procedures in patients with synchronous bilateral Wilms tumors: a report from the National Wilms Tumor Study Group. J Pediatr Surg 1996;31:1020-5.

[8] Coppes MJ, Muoi A, Beckwith JB, et al. Factors affecting the risk of contralateral Wilms tumor development: a report from the National Wilms Tumor Study Group. Cancer 1999;85:1616-24.

[9] Kullendorff CM, Wiebe T. Bilateral Wilms tumor. Pediatr Surg Int 1999;15:46-9.

[10] Paya K, Horcher E, Lawewnz K, et al. Bilateral Wilms tumor—surgical aspects. Eur J Pediatr Surg 2001;11:99-104.

[11] Desai D, Nicholls G, Duffy PG. Bench surgery with autotransplantation for bilateral synchronous Wilms tumor: a report of three cases. J Pediatr Surg 1999;34:632-4.

[12] Green DM. The treatment of stage I-IV favorable histology Wilms tumor. J Clin Oncol 2004;22:1366-72.

[13] Greenberg M, Burnweit C, Weitzman S, et al. Preoperative chemotherapy for children with Wilms tumor. J Pediatr Surg 1991;26:949-56.

Pediatr Surg Int (2006) 22:891–896
DOI 10.1007/s00383-006-1762-0

ORIGINAL ARTICLE

Association between the HER2 expression and histological differentiation in Wilms tumor

M. Salem · Y. Kinoshita · T. Tajiri · R. Souzaki · K. Tatsuta · M. Higashi · T. Izaki · K. Kohashi · M. Tsuneyoshi · T. Taguchi

Published online: 25 August 2006

Abstract Human epidermal growth factor receptors (HER) play a critical role in the branching morphogenesis of renal tubules. In the current study, we analyzed the expression of HER2 in Wilms tumor and assessed the role of this gene in the tumorgenesis of Wilms tumor. During the period from 1960 to 2005, 40 patients with Wilms tumor were treated in our department. Twenty-four of those patients (except those with clear cell sarcoma of the kidney and malignant rhabdoid tumor of the kidney) were collected and assessed. The histological component of each Wilms tumor was divided into three categories (epithelial, blastemal, and mesenchymal) and the extent of HER2 protein expression was analyzed immunohistochemically. The normal kidney tissue accompanied with 12 cases of Wilms tumor was also examined. In the normal kidney, HER2 showed a strong immunoreactivity in the cell membranes of the collecting tubules and in the endothelial cells. Of 24 cases, 15 cases showed an epithelial component, while 24 cases had a blastemal component and 21 cases had a mesenchymal component, respectively. Among the 15 specimens with epithelial cell differentiation, eight (53.3%) showed HER2 immunoreactive epithelial cells. HER2 immunoreactive blastemal cells were present in 11 (45.8%) of 24 specimens with blastemal cells. On the other hand, only 3 (14.3%) of 21 specimens containing mesenchymal cells showed HER2 immunoreactivity. These results suggest that the extent of HER2 expression is associated with epithelial differentiation in Wilms tumor. These histological findings may therefore help to explain the development of Wilms tumor from the standpoint of histological differentiation.

Keywords Wilms tumor · HER2 · Epithelial · Blastemal · Mesenchymal

M. Salem · Y. Kinoshita (✉) · T. Tajiri · R. Souzaki · K. Tatsuta · M. Higashi · T. Izaki · T. Taguchi
Department of Pediatric Surgery, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
e-mail: kinoppy@pedsurg.med.kyushu-u.ac.jp

K. Kohashi · M. Tsuneyoshi
Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Introduction

Wilms tumor is a pediatric malignancy and one of the most common solid tumors occurring in children [1]. The biologic behavior is difficult to predict on the basis of histopathologic findings alone [2].

Human epidermal growth factor receptor 2 (HER2), which is encoded by the c-erb-B2 gene, is a transmembrane glycoprotein belonging to a family of four receptors for tyrosine kinases that mediate cellular proliferation, differentiation, and survival through the binding of growth factor ligands [3–6]. HER2 was originally isolated from an ethylnitrosourea-induced rodent neuroblastoma [7] and it has subsequently been identified in other human malignant solid tumors [8–10]. Both in vitro and in vivo studies have demonstrated the gene amplification or protein overexpression of HER2 to induce tumorigenesis, to increase the metastatic potential, and to promote chemoresistance [11, 12]. The dysregulation of these receptors is linked

to multiple features of malignant tumors, including a loss of cell cycle control, resistance to apoptotic stimuli, invasiveness, chemo-resistance, and the induction of angiogenesis [13]. On the other hand, HER2 has also been demonstrated to play a critical role in the branching morphogenesis of renal tubules [14].

In the current study, we analyzed the expression of HER2 in Wilms tumor and assessed the role of this gene in the tumorgenesis of Wilms tumor.

Materials and methods

Materials

During the period from 1960 to 2005, 40 patients with Wilms tumor were treated in our department. The cases with Wilms tumors, except for clear cell carcinoma of the kidney (CCSK) and malignant rhabdoid tumor of the kidney (MRTK), were collected, and 24 of them were eligible for evaluation in the current study. The patients were followed regularly and all data concerning the diagnosis, treatment, and follow-up were stored in a database. Twenty-three cases had a favorable histology, while one case had an unfavorable histology showing anaplastic features. The normal kidney tissue specimens from 12 of the cases were also examined.

Light microscopic findings

All nephrectomy specimens from the 24 cases were fixed in 10% formalin and embedded in paraffin. The hematoxylin and eosin-stained slides were available and reviewed by pediatric pathologists (K.K. and M.T.). The histological components of each Wilms tumor were divided into three categories (epithelial, blastemal, and mesenchymal). Furthermore, according to the predominant cell type, a detailed phase classification (monophasic, biphasic, and triphasic) was performed.

Immunohistochemical analysis

An immunohistochemical study was performed using polyclonal rabbit anti-human c-erbB-2 oncoprotein antibody [Dako A0485 (Hamburg, Germany), dilution: 1:150]. Next, sections measuring four micrometers in thickness were placed onto silane-treated slides and deparaffinized, before being rehydrated with xylene and a graded series of ethanol. After being pretreated with heat-induced antigen-retrieval method in a citrate buffer, the sections were incubated with the primary antibody overnight at 4°C. The subsequent development of antibody-bridge labeling was made using the streptavidin biotin peroxidase method (Histofine SAB-PO Kit, Nichirei, Tokyo, Japan) with hematoxylin counterstaining.

Human epidermal growth factor receptor 2 staining was scored based on the percentage of positive cells in a particular area (without any knowledge of the clinical outcome of the patients) according to the standard criteria (Table 1) [12]. Breast adenocarcinoma served as positive control. We next correlated the HER2 expression with the clinical course of the patients. This study was performed according to the "Ethical guidelines for clinical research" published by Ministry of Health, Labour and Welfare of Japan on July 30, 2003.

Results

Histological phase and predominant cells

Among the 24 cases, 12 cases showed a triphasic pattern composed of epithelial, blastemal, and mesenchymal elements. The other 12 cases showed a biphasic pattern composed of epithelial and blastemal elements, or balastemal and mesenchymal elements. None of the cases showed a monophasic pattern. According to the predominancy, blastemal cells were predominant in 19 of 24 cases. According to the elements of cell type, epithelial cells were identified in 15 cases, while blastemal cells were seen in 24 cases and mesenchymal cells were found in 21 cases, respectively (Table 2).

Table 1 Score for HER2 overexpression

Score	Assessment	Staining pattern
0	Negative	No staining or membrane staining is observed in <10% of tumor cells
1+	Negative	Faint or barely perceptible membrane staining is detected in >10% of tumor cells; the cells are only stained in part of their membrane
2+	Weak to moderate positive	Weak to moderate complete membrane staining is observed in >10% of tumor cells
3+	Strong positive	Strong complete membrane staining is observed in >10% of tumor cells

From Ref. [12]