stitutively activated by *ALK* gene amplification in three neuroblastoma cell lines, indicating a novel mechanism of activation of ALK kinase in malignancies.[27] In this study, amplification of the *ALK* gene was detected in primary neuroblastoma tissues for the first time. This suggests that activated ALK kinase plays a real role in the pathophysiology of neuroblastoma, such as giving a more malignant phenotype to the tumors by perturbing signal transduction. Recently, Motegi et al[33] showed that ALK transmits both mitogenic and differentiation signals, and that the MAPK pathway plays an important role in these effects in SK-N-SH cells without *ALK* gene amplification. Together with the fact that activated ALK surpasses regulation by other RTKs in cell lines with *ALK* gene amplification,[27] our new results showing apoptotic changes caused by the suppression of activated ALK protein clearly demonstrate the dominant role of ALK kinase in the survival of the *ALK*-amplified type of neuroblastoma.

The frequency and copy numbers of gene amplification of ALK were significantly lower in neuroblastic tumors compared with neuroblastic cell lines. Remarkable amplification of the *ALK* gene was detected in 1 tumor tissue of 85 tumor samples examined. Three neuroblastoma cell lines with *ALK* amplification had more than 30 copies of *ALK*, whereas primary neuroblastoma containing *ALK* gene amplification had within a range of 2 to 10 copies. This may be due to underestimation of the copy number in the tumor cells because of contamination of stromal cells and lymphocytes into the tumor tissues.[34,35] There may also be a mechanism in which cells with a higher copy number of *ALK* become the major population during the establishment of cell lines because of their growth advantage. Immunohistochemical analysis demonstrated, however, universal cytoplasmic expression of ALK in a wide range of neuroblastoma tumor samples, suggesting some transcriptional or posttranslational regulation of the ALK amount might exist in neuroblastoma cells. Although, due to the condition of the samples, we were unable to obtain information on the copy numbers of the *ALK* gene as for the samples used in the immunohistochemical analysis, further immunohistochemical screening may reveal neuroblastoma tissues with an outstanding amount of ALK protein because of gene amplification.

The N-*myc* gene was also amplified in this tumor and in all three cell lines with *ALK* amplification (NB-39-nu, Nagai, and NB-1). N-*myc* is located on 2p24.3 and *ALK* is on 2p23.2, suggesting that there is a tendency to synchronic amplification between N-*myc* and *ALK*. We were unable to conclude that there was an association between ALK amplification and prognosis mainly due to the limited number of positive samples and the short-term follow-up. Moreover, the *ALK* gene locus appears too far from the N-*myc* gene locus to be within a single amplicon. Further analysis in a greater number of samples with longer follow-up is necessary.

The activation of ALK results in hyperphosphorylation of ShcC in neuroblastoma cells, and NB-39-nu cells treated with ALK-siRNAs show suppressed tyrosine phosphorylation of ShcC, followed by apoptotic changes to these cells, suggesting that ShcC is a physiological substrate of the activated ALK kinase and that the ALK-ShcC pathway dominantly controls the survival of NB-39-nu cells even with the existence of other RTKs, such as EGFR, TrkA, and Ret. In neuronal cells, both ShcB (Sli/SCK) and ShcC (Rai/N-Shc) can bind activated RTKs, including the EGFR and Trk receptor.[36–39] Mice lacking both ShcB and ShcC exhibit a significant loss of sympathetic neurons, suggesting that ShcB and ShcC act in supporting sympathetic development and survival.[28] A recent study also showed that ShcC is a physiological substrate of Ret kinase and that it exerts a prosurvival function in neuronal cells.[40] Although high levels of TrkA expression correlate with a favorable outcome of neuroblastoma patients,[20] TrkA expression was significantly high in NB-39-nu and Nagai, which derive from tumors with a poor prognosis. This discrepancy may also be explained by the overwhelming control of cell survival by ALK kinase in these cell lines. Neuronal apoptosis is regulated through the action of critical protein kinase cascades, such as the phosphatidylinositol 3-kinase/Akt pathway and the Ras-MAPK pathway.[41,42] Apparently, neither pathway is properly controlled by EGF or nerve growth factor in NB-39-nu cells or Nagai cells.[27] Here, we also demonstrated that the suppression of activated ALK blocks MAPKs and Akt in these cells, resulting in apoptosis. On the other hand, the activity of MAPKs and Akt was not reduced by the suppression of a single copy of *ALK* in SK-N-MC cells. These results suggest that activation of ALK kinase completely remodeled the cellular signaling transduction pathways through ShcC so that cell survival entirely depended on signals originating from ALK kinase.

In conclusion, phosphorylation of several signaling molecules and cancer survival might be under the control of activated ALK kinase when gene amplification of ALK is as significant as in NB-39-nu cells, although the frequency of gene amplification in neuroblastoma tissues is not high. Cytoplasmic expression of ALK in neuroblastoma cells may suggest distinct function of this kinase in cell proliferation and survival. These findings further suggest that activated ALK kinase will be indispensable information for prognosis and treatment of neuroblastoma although the frequency is low.

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Aberrant methylation of *FBN2* in human non-small cell lung cancer

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Summary FBN2, a large modular extracellular matrix glycoprotein, is known to be a key component of human elastic fiber. A loss of *FBN2* expression due to promoter methylation was recently identified in pancreatic cancer. We examined *FBN2* expression by reverse transcription PCR and aberrant methylation of *FBN2* by methylation specific PCR in lung cancer cell lines. Aberrant methylation of *FBN2* was present in 55% (6 of 11) of non-small cell lung cancer (NSCLC) cell lines, but it absent in small cell lung cancer cell lines. The concordance between loss of expression and aberrant methylation of *FBN2* was 88% (14 of 16) in the cell lines. *FBN2* expression was restored after treatment with the demethylating agent, 5-aza-2′-deoxycytidine in all six cell lines tested that lacked *FBN2* expression. Among primary NSCLC, 49% (62/126) of cases had *FBN2* methylation, but only 7% (5/69) of the corresponding nonmalignant lung tissues had it. Although *FBN2* methylation was detected even in patients with early stage disease, it occurred frequently in large tumors ($p=0.022$), with nodal metastasis ($p=0.037$), or with advanced stages of NSCLC ($p=0.014$). Methylation and silencing of *FBN2* in tumor cells may play an important role in carcinogenesis, invasion, and metastasis of NSCLC.

1. Introduction

It is well known that genetic abnormalities of proto-oncogenes and tumor suppressor genes (TSGs) are frequently involved in lung cancer pathogenesis.

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The mechanism for inactivation of TSGs is gradually becoming more clearly understood. Epigenetic inactivation of certain TSGs by aberrant promoter methylation is frequently observed in lung cancer and seems to play an important role in the pathogenesis of this cancer [1–4]. In addition, the study of the loss of heterozyosity (LOH) which is also involved in the carcinogenesis of lung cancer, showed that correlations between LOH on different chromosomes suggested previously unknown genetic interactions for lung cancer development [5]. So, whereas the DNA methylation of multiple genes has been studied in lung cancer [6–8], further studies of epigenetic alternation are still needed to clarify fully the biological mechanism of lung cancer.

Fibrillin 2 (FBN2), an extracellular matrix protein, is associated with elastic fibers in several tissues and is believed to serve as a ligand for alphavbeta3 integrin, the latter being a known morphogen. *FBN2* was first expressed in the mesenchyme and at the epitheliomesenchymal interface. Latter, its expression was intensified and was confined around the tracheobronchial airways. Fibrillin-2 antisense oligodeoxynucleotide can induce dysmorphogenesis of the lung explants. FBN2 plays a key role in lung development [9].

Recently, the loss of *FBN2* expression due to promoter methylation was identified in pancreatic cancer cell lines by means of high-throughput microarray analysis [10]. Of the 12 genes silenced by methylation of 5′ regions, FBN2 was methylated in about 75% of the samples, which is a much higher proportion than for the other genes. This gene maps to 5q23-q31, a locus frequently showing allelic imbalance in lung cancer, and was speculated to act as a TSG [5]. This prompted us to examine the methylation status of *FBN2* in lung cancers. We examined methylation by methylation specific PCR (MSP), and the mRNA expression of *FBN2* by reverse transcription PCR (RT-PCR), in lung cancer cell lines, and analyzed the methylation status of primary lung cancers, and then correlated this with the clinico-pathological features.

2. Materials and methods

2.1. Cell lines and clinic samples

Eleven non-small cell lung cancer (NSCLC) and five small cell lung cancer (SCLC) cell lines were used in this study. These cell lines were established and provided by Dr. Adi F. Gazdar of University of Texas (UT) Southwestern Medical Center. Cell lines having the prefix NCI were established at the National Cancer Institute, while those with the prefix HCC were established at UT Southwestern Medical Center. They were grown in RPMI-1640 medium supplemented with 5% fetal bovine serum and incubated in 5% CO_2 at 37 °C. Nonmalignant human bronchial epithelial cells (NHBEC) were cultured as reported previously [11], and normal tracheal RNA was obtained from Clontech (Palo Alto, CA).

Surgically resected specimens of 126 patients with primary lung cancer and 69 adjacent lung tissues were obtained from Chiba Cancer Center, Japan, after obtaining Institutional Review Board approval and informed consent had been granted. Samples were immediately frozen and stored at −80 °C until used. The clinical characteristics of these patients are detailed in Table 1.

2.2. RNA preparation and RT-PCR

FBN2 mRNA expression was examined by RT-PCR. Total RNA was obtained from these cell lines (NHBEC, 11 NSCLC and 5 SCLC cell lines) by the single-step method. The reverse transcription reaction was performed on 5 μg of total RNA with the SuperScript II First-Strand Synthesis using oligo(dT) primer System (Life Technologies Inc.), and aliquots of the reaction mixture were used for the subsequent PCR amplification. Expression of β-actin was used as an internal control to confirm the success of the reverse transcription reaction. The forward PCR amplification primer of *FBN2* was 5′-GGCGAGGACAGCAGGAC-3′, and the reverse primer 5′-TGATATTTGCCCACTGGAACA-3′. The forward PCR amplification primer of β-actin was 5′-CAACTGGGACGACATGGAGA-3′, and the reverse primer 5′- ACGTACATGGTGGGGTGTTG-3′. These primer sequences were identical to the human target genes as was confirmed by BLAST searches. PCR products were analyzed on 2% agarose gels stained with ethidium bromide. NHBEC and normal tracheal cells were used as normal controls for RT-PCR.

2.3. 5-Aza-2′-deoxycytidine (5-Aza-CdR) treatment

Six tumor cell lines with negative gene expression were incubated in culture medium with 1 μM of the demethylating agent 5-aza-dC (Sigma-Aldrich, St. Louis, Mo) for 6 days, with medium changes on days one, three and five. Cells were harvested and RNA was extracted at day 6.

Table 1 Clinical characteristics and *FBN2* methylation of lung cancer patients

Clinical factors	No. of cases	No. of *FBN2* methylation (%)	*p*-value[a]
Gender			
Male	73	38 (52)	NS
Female	53	24 (45)	
Age			
≤65[b]	58	25 (43)	NS
>65	68	37 (54)	
Smoke			
Never	47	20 (43)	NS
Smoker	79	42 (53)	
Histology			
Adenocarcinoma	92	49 (53)	NS[c]
Squamous cell carcinoma	30	11 (37)	
Others (ad-sq, Large)	4	2 (50)	
pT			
T1	53	20 (38)	0.022
T2, 3 4	73	42 (58)	
pN			
N0	76	32 (42)	0.037
N1, 2, 3	50	30 (60)	
pStage			
I	54	20 (37)	0.014
II, III, IV	72	42 (58)	

[a] Fisher's exact probability test.
[b] Devided into two groups by median age.
[c] Adenocarcinoma vs. squamous cell carcinoma ad-sq, adeno-squamous cell carcinoma; NS, not significant.

2.4. DNA preparation, bisulfite modification and MSP

Genomic DNA was obtained from lung cancer cell lines, cultured nonmalignant cells, primary tumors and adjacent nonmalignant tissues by digestion with proteinase K (Life Technologies, Inc.), followed by phenol/chloroform (1:1) extraction [12]. One microgram of genomic DNA was further subjected to bisulfite treatment following the protocol of the EZ DNA Methylation Kit (Zymo Research). The modified DNA was used as a template for MSP. DNA methylation patterns in the CpG island of *FBN2* were determined by the method of MSP as reported previously [10]. Primer sequences of *FBN2* for the unmethylated reaction were: 5′-TATGGGAAT -TTGTTGAGTTTTGT-3′ (sense), and 5′-AACCAACAACCCCAAACA-3′ (antisense), which amplify a 171 bp product. Primer sequences of *FBN2* for the methylated reaction were: 5′-GGGAATTCGTCGAGTTTTGC-3′ (sense), and 5′-AACCGACAACCCCGAACG-3′ (antisense), which amplify a 168 bp product. Universal Methylated DNA (Chemicon, CA) which was subjected to bisulfite treatment was used as a positive control for methylated alleles. Controls without DNA were included in each assay. Nine microlitre of each PCR product was loaded on 2% agarose gels stained with ethidium bromide. Results were confirmed by repeating the bisulfite treatment and MSP for all samples.

2.5. Statistical analysis

The differences of methylation between the two groups were analyzed by using Fisher's exact test. Survival was calculated from the date of initial diagnosis until death or the date of the last follow-up. Survival was analyzed, according to the Kaplan-Meier method, and differences in their distribution were evaluated by means of the log-rank test. A probability value of p less than 0.05 was regarded as statistically significant. All Ps are two-sided.

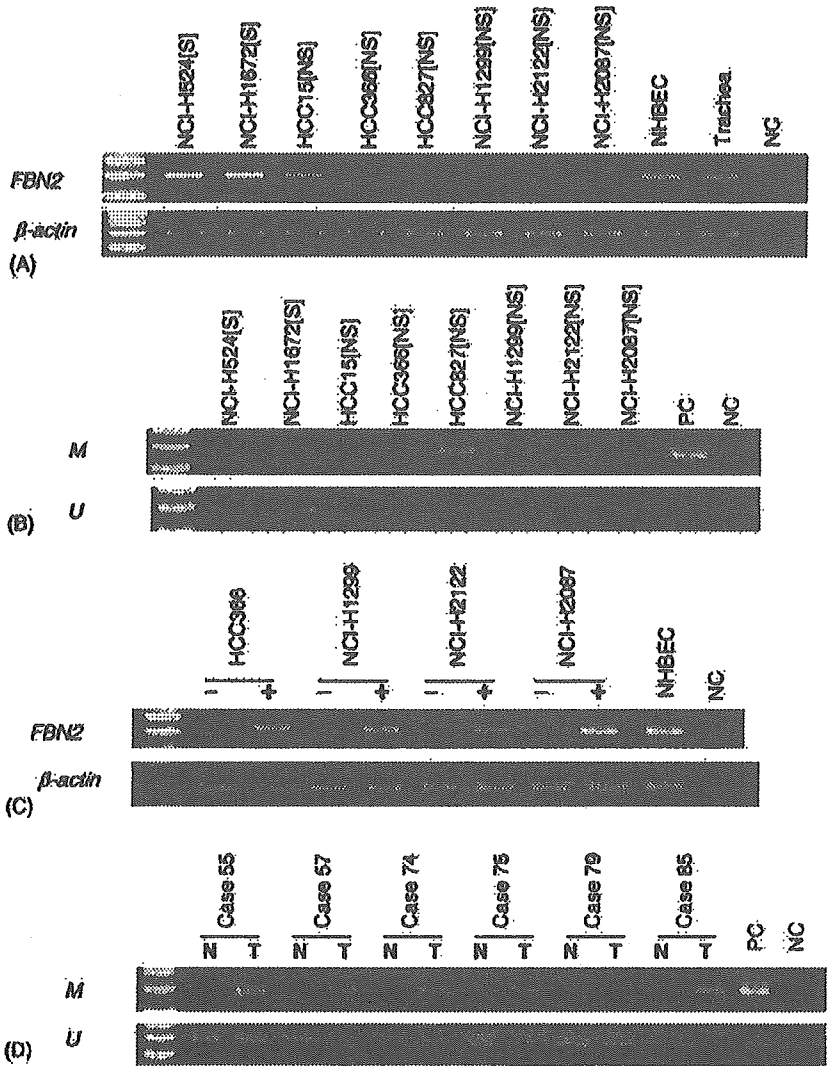

Fig. 1 (A) Representative examples of RT-PCR assay for *FBN2* RNA expression in NSCLC and SCLC cell lines. Expression of β-actin was used as a control for cDNA normalization. NHBEC and trachea were used as positive controls; NC, negative control. [NS], NSCLC; [S], SCLC. Lanes that do not show a band represent samples with loss of expression. (B) Methylation analysis of *FBN2* in cell lines. Lane U, amplified product with primers recognizing an unmethylated sequence (171-bp PCR product); Lane M, amplified product with primers recognizing a methylated sequence (168-bp PCR product). PC, positive control; NC, negative control. C, reexpression of *FBN2* after treatment with 5-Aza-2′-deoxycytidine (5-Aza-CdR). The expression of *FBN2* lost in those cell lines can be restored after treatment with 5-Aza-CdR. −, cell line without 5-Aza-CdR; +, cell line with 5-Aza-CdR; NHBEC was used as the positive control. (D), Representative examples of methylation analysis of *FBN2* in tumor specimens. N, nonmalignant lung tissue; T, tumor.

3. Results

3.1. Expression of *FBN2* in cell lines

FBN2 expression was examined by RT-PCR, and representative examples are shown in Fig. 1. Expression of *FBN2* was present in NHBEC and normal trachea. However, loss of *FBN2* expression was observed in 50% (8/16) of lung cancer cell lines, respectively, in 64% (7/11) of NSCLC cell lines, and in 20% (1/5) of SCLC cell lines.

3.2. Aberrant methylation of *FBN2* in cell lines

Detailed results of the aberrant methylation of *FBN2* in cell lines are showed in Fig. 1. Aberrant methylation was absent in NHBEC, but was observed

in 38% (6/16) of lung cancer cell lines, in 55% (6/11) of NSCLC cell lines, but in no SCLC cell lines. Only two cell lines (NCI-H524 and HCC15) demonstrated loss of expression and lack of methylation of *FBN2*. The concordance between gene expression and methylation of *FBN2* was 91% (10/11) in the NSCLC cell lines, and 80% (4/5) in the SCLC cell lines (overall concordance: 88%).

3.3. 5-Aza-CdR treatment

To confirm that the promoter methylation was responsible for silencing the *FBN2* expression, we treated methylated NSCLC cell lines (HCC366, HCC827, NCI-H1299, NCI-H2087, NCI-H2122, and NCI-H2887) that showed loss of *FBN2* expression with the demethylating agent 5-Aza-CdR. *FBN2* expression was restored after the treatment in all six cell lines tested (Fig. 1).

3.4. Aberrant methylation of *FBN2* in primary lung cancers

FBN2 methylation of primary tumors and corresponding nonmalignant tissues are detailed in Table 1 and representative samples are illustrated in Fig. 1. *FBN2* methylation was observed in 49% (62/126) of tumors, but in only 7% (5/69) of corresponding nonmalignant tissues. Methylation was tumor-specific when compared with that of corresponding nonmalignant lung tissue ($p < 0.0001$).

FBN2 methylation with clinico-pathological features was also examined. There were no significant correlations in gender, age, smoking history (ever versus never smoked). The aberrant methylation of *FBN2* gene was present in 53% (49/92) cases of adenocarcinoma, and in 37% (11/30) of the squamous cell carcinoma cases. The difference between these results is not significant ($p = 0.09$). Because the number of squamous cell carcinomas and other histologies was small, we carried out a further study on the whole population of patients, unclassified by histology. The frequency of *FBN2* methylation was higher in the later T stages (T2, 3, 4) than in T1 ($p = 0.022$), higher in the later N stages (N1, 2, 3) than in N0 ($p = 0.037$), and in the later stages (II, III, IV) than in stage I ($p = 0.014$). However, *FBN2* methylation status did not correlate with survival ($p = 0.37$, log-rank test). The Cox proportional hazards model was also used to evaluate the effects of *FBN2* methylation with other explanatory variables on survival time, but the *FBN2* methylation was not a significant independent factor (data not shown).

4. Discussion

Tumor invasion is one of the earliest steps in the multistep process of metastasis and is characterized by cancer cells invading and breaking the basement membrane or other components of the extracellular matrix. Therefore, alteration of the extracellular matrix molecule is important for the development of malignant tumors [13]. FBN2, a large modular extracellular matrix glycoprotein found in many vertebrate organ systems, is known to be a key component of elastic fiber. Recently, Hagihara et al. demonstrated that *FBN2* is frequently methylated in pancreatic cancer [10]. However, there is no report on the role of FBN2 in lung cancer. To understand the role of *FBN2* gene in lung cancer, we examined the expression of *FBN2*. It was expressed in tracheal cells, and cultured airway epithelial cells, whereas the lung cancer cell lines showed a loss of 50% of the expression. Treatment with 5-Aza-CdR restored the expression of the gene in RT-PCR-negative cell lines, indicating that methylation is a major mechanism of transcriptional silencing of the gene. Also, tumor-specific methylation of *FBN2* gene was present in 49% of NSCLC. Although other mechanisms for disruption of the extracellular matrix exist, such as inactivation of laminin5-encoding genes [14–16] or overexpression of matrix metalloprotienases [17,18], aberrant methylation of *FBN2* may be one of those participating in the process of tumor progression.

In our study, two cell lines showed a loss FBN2 expression with a lack of aberrant methylation. This may have been due to other mechanisms of inactivating TSGs, such as loss of heterozygosity, point mutations, and homozygous deletions [19]. Also, we found FBN2 methylation in some of the matched nonmalignant lung tissues. Possible explanations for detecting methylated alleles in the nonmalignant lung tissues are that they may represent premalignant changes [20], or be related to age [21] or smoking [22].

In this study, 49% of *FBN2* methylation was associated with an increase in tumor size, lymph node involvement, and advanced stages. Although further study will be required in order to understand the role of FBN2 in the pathogenesis of advanced lung cancer, our data suggested that it is advantageous for tumor invasion and metastasis to downregulate *FBN2* gene of cancer cells. There are several reports about the correlation between the aberrant methylation of TSG and the progression of lung cancer [23,24]. *FBN2* may be added to the list of progression associated methylation genes of lung cancer because of the high frequency of methylation correlated significantly with progression. To

our knowledge, this is the first report that demonstrating the methylation of *FBN2* promoter in lung cancer and the correlation between *FBN2* methylation and lung cancer progression.

Our data did not show a significant association between *FBN2* methylation and patient survival. The lack of significance in the relationship of methylation to survival outcome may result from the limited number of patients taking part in our study, or on other factors.

In conclusion, we demonstrated frequent inactivation of *FBN2* gene through aberrant methylation of the promoter in NSCLC cell lines. We also found methylation of *FBN2* frequently in primary NSCLC; it correlated with the progression of the tumor from early to late stage disease. Aberrant methylation of *FBN2* gene appears to be an important factor in the pathogenesis of invasive NSCLC. Our findings of a frequent acquired tumor-related epigenetic alteration favor the candidacy of *FBN2* as a TSG.

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ORIGINAL REPORT

Prediction of *MYCN* Amplification in Neuroblastoma Using Serum DNA and Real-Time Quantitative Polymerase Chain Reaction

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ABSTRACT

Purpose

MYCN amplification (MNA) indicates a poor prognosis in neuroblastoma (NB) and is routinely assayed for therapy stratification. We aimed to develop a diagnostic tool to predict *MYCN* status using serum DNA, which, in cancer patients, predominantly originates from tumor-released DNA.

Patients and Methods

Using DNA-based real-time quantitative polymerase chain reaction, we simultaneously quantified *MYCN* (2p24) and a reference gene, *NAGK* (2p12), and evaluated *MYCN* copy number as an *MYCN/NAGK* (*M/N*) ratio in 87 NB patients whose *MYCN* status had been determined by Southern blotting. Of these patients, 17 had *MYCN*-amplified NB, and 70 had nonamplified NB.

Results

The serum *M/N* ratio in the MNA group (median, 199.32; range, 17.1 to 901.6; 99% CI, 107.0 to 528.7) was significantly ($P < .001$) higher than the ratio in the non-MNA group (median, 0.87; range, 0.25 to 4.6; 99% CI, 0.82 to 1.26; Mann-Whitney *U* test). The sensitivity and specificity of the serum *M/N* ratio as a diagnostic test were both 100% when the serum *M/N* ratio cutoff was set at 10.0. Among six MNA patients whose clinical courses were followed, the serum ratios decreased to the normal range in the patients in remission (n = 3), whereas the ratios increased to high levels in the patients who relapsed (n = 2) or failed to achieve remission (n = 1).

Conclusion

Measurement of the serum *M/N* ratio seems to be a promising method for accurately assessing *MYCN* status in NB, although a larger set of patients needs to be examined to confirm this result.

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INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid tumor in children and is characterized by a wide range of clinical behaviors, from spontaneous regression to rapid progression with a fatal outcome. The clinical heterogeneity has been reported to be associated with a variety of biologic features of NB. One such aberration, *MYCN* amplification (MNA; ie, creation of multiple copies of the *MYCN* gene in the nuclei of tumor cells), is strongly associated with rapid tumor progression and a poor outcome. MNA is detected in 4% of patients in the early stages of NB, 8% of patients in stage 4S, and approximately 30% of patients in advanced stages. Currently, assessment of *MYCN* status is essential for determining therapy stratification in NB.[1-6] Having rapid access to selected biologic data for each tumor has become increasingly important in

routing patients to appropriate therapies. Several years ago, fluorescence in situ hybridization (FISH) replaced Southern blotting as the most accurate and timely way of evaluating tumors for MNA. Using FISH, the turnaround time for results was shortened from weeks to days, making its use in clinical trials realistic.

In this study, we describe a real-time polymerase chain reaction (PCR) method for evaluating *MYCN* status that shortens the turnaround for results to just a few hours. Furthermore, to facilitate the evaluation of *MYCN* status of tumors, we used serum DNA for the PCR template, which, in cancer patients, predominantly consists of tumor-released DNA.[7] Quantification of serum DNA has also been proposed as a screening tool for early detection of lung cancer.[8] Several groups were able to detect tumor-related aberrations, such as loss of heterozygosity and mutations in the *p53* gene, using the serum DNA of patients with a malignant tumor.[9-11]

Recently, Combaret et al[12] reported that high levels of *MYCN* DNA were present in the peripheral blood of patients with *MYCN*-amplified NB. However, they evaluated serum *MYCN* (2p24) dosage based on PCR without a reference gene, so their assay could be influenced by the quality of the template DNA or a numerical change of chromosome 2. To avoid these problems, we used DNA-based real-time quantitative PCR and a single copy reference gene, the *N-acetylglucosamine kinase* gene (*NAGK*; 2p12), so that *MYCN* copy number per chromosome 2 could be evaluated as the *MYCN/NAGK* (*M/N*) ratio. *NAGK* was chosen because it is on the same chromosome as *MYCN* but sufficiently distant from the region spanned by the *MYCN* amplicon (2p12 *v* 2p24)[13] that a numerical change in chromosome 2 would not affect the *M/N* ratio. The diagnostic performance of the test was evaluated in patients with an NB whose *MYCN* status had been determined by Southern blotting.

PATIENTS AND METHODS

Subjects

Eighty-seven patients diagnosed with NB at the Hospital of the Kyoto Prefectural University of Medicine and Chiba Cancer Center Research Institute were enrolled onto this study with the informed consent of their parents. The studies were conducted under research protocols approved by each institutional review board. At the time of diagnosis, 44 patients were younger than 1 year, and 43 were between 1 and 13 years of age. Seventeen of the patients had MNA, and 70 patients did not have MNA, as determined by Southern blotting. According to the International Neuroblastoma Staging System,[4] the 17 children with MNA included one patient each in stage 1 and 2B, two in stage 3, and 13 in stage 4, whereas the 70 children without MNA included 22 in stage 1, 18 in stage 2A and 2B, five in stage 4S, seven in stage 3, and 18 in stage 4.

Twelve of the 17 patients with MNA and 33 of the 70 nonamplified patients were also analyzed by dual-color FISH technique, as previously described,[14] using an *MYCN* probe (pNb101) and a chromosome 2 centromere probe (D2Z). FISH results of these patients were consistent with the Southern blotting results, although three of the patients who were diagnosed as non-MNA by Southern blotting were found to have one to four extra copies of the *MYCN* gene relative to the chromosome 2 centromere number by FISH. This low level of amplification has been defined as *MYCN* gain, which is an intermediate stage between MNA and non-MNA.[15] Because the prognostic significance of *MYCN* gain is still unclear, these patients were classified as non-MNA according to the Southern blotting results.

Sample Preparation

Tumor specimens were surgically resected and immediately stored at −80°C. Peripheral blood was obtained from each patient before any therapy and surgery. To avoid contamination of serum DNA by the DNA from WBCs, we prepared serum exclusively from the liquid fraction of clotted blood after centrifugation at 1,000 × *g* for 10 minutes and stored it at −20°C until DNA extraction.

DNA Isolation

DNA was extracted from tissues and serum samples by using the QIAmp tissue and blood kits (Qiagen, GmbH, Hilden, Germany), respectively, according to the manufacturer's protocols. For each patient, 200 μL of the stored serum was used for extraction of free DNA.

Real-Time Quantitative PCR

TaqMan PCR was performed using the ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR mixture contained TaqMan universal PCR master mix (Applied Biosystems), 200 nmol/L of each primer, and 100 nmol/L of fluorogenic probe. The principle of the TaqMan analysis has been described previously in detail.[16-18] In addition to the *MYCN* sequence, *NAGK* (GenBank accession No. NM 017567) located at 2p12 was simultaneously measured as a single-copy reference gene. The sequence of primers and the TaqMan probe used for *MYCN* and *NAGK* are as follows: *MYCN* forward, 5′-GTGCTCTCCAATTCTCGCCT-3′; *MYCN* reverse, 5′-GATGGCCTAGAGGAGGGCT-3′; *MYCN* probe, 5′-FAM-CACTAAAGTTCCTTCCACCCTCTCCT-TAMRA-3′; *NAGK* forward, 5′-TGGGCAGACACATCGTAGCA-3′; *NAGK* reverse, 5′-CACCTTCACTCCCACCTCAAC-3′; and *NAGK* probe, 5′-VIC-TGTTGCCCGAGATTGACCCGGT-TAMRA-3′. All PCR reactions were performed with one cycle of 95°C for 5 minutes, followed by PCR amplification with 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curves were constructed in each PCR run with four-fold serial dilutions containing 20, 5, 1.25, 0.3125, and 0.078125 ng/μL of a healthy donor's DNA in addition to 20 ng/μL of salmon sperm DNA, and the dosages of the target genes in each sample were interpolated using these standard curves. The *MYCN* copy number of a sample of DNA was determined by the ratio of the *MYCN* dosage to the *NAGK* dosage (*M/N* ratio). Copy numbers were expressed as the average of two measurements.

Effect of WBC Contamination

To assess the effect of WBC contamination in serum samples on the serum *M/N* ratio, we measured the serum *M/N* ratio using DNA extracted from a series of WBC-contaminated serum samples. The samples were prepared by adding 0, 1×10, 1×10^2, 1×10^3, 1×10^4, and 1×10^5 of WBCs from a healthy donor to 200 μL of serum from a *MYCN*-amplified patient.

Statistical Methods

The difference in the serum *M/N* ratio between the MNA and non-MNA groups was assessed using the Mann-Whitney *U* test. $P < .05$ was judged as significant.

RESULTS

Serum M/N *Ratio As a Predictor of* MYCN *Status of Tumor*

Serum *M/N* ratios could be determined in approximately 4 hours by real-time quantitative PCR. Figure 1 shows the distribution of the serum *M/N* ratio in the MNA and non-MNA groups at the time of diagnosis. The serum *M/N* ratio in the MNA group (n = 17; median, 199.32; range, 17.1 to 901.6; 99% CI, 107.0 to 528.7) was significantly ($P < .001$) higher than the ratio in the non-MNA group (n = 70; median, 0.87; range, 0.25 to 4.6; 99% CI, 0.82 to 1.26). In fact, there was no overlap between the two groups in the limited number of patients examined in this study. As a cutoff for the serum *M/N* ratio to distinguish between MNA and non-MNA patients, we empirically chose a value of 10, which was in the middle of the two ranges. With this value, the sensitivity and specificity of the serum *M/N* ratio as a diagnostic test to distinguish patients with MNA from those without MNA were both 100% for our limited number of patients. That is, the serum *M/N* ratio was in complete agreement with the Southern blotting results. The positive and negative predictive values were 100%. The serum *M/N* ratios were also consistent with results obtained by FISH for 45 of the patients (FISH analyses were performed in 12 of the 17 MNA patients and in 33 of the 70 nonamplified patients). Three of the patients who had one to four extra copies of the *MYCN* gene relative to chromosome 2 centromere number, as determined by FISH, also had slightly elevated serum *M/N* ratios (2.5, 3.3, and 4.6).

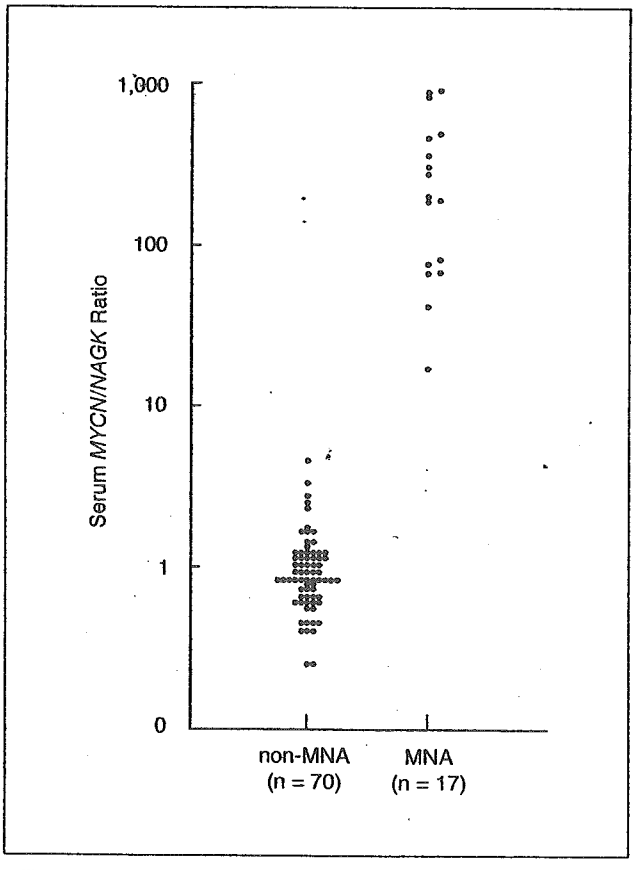

Fig 1. A scatter plot of serum *MYCN/NAGK* ratio in patients with *MYCN*-amplified (MNA) and nonamplified (non-MNA) neuroblastoma. The serum *MYCN/NAGK* ratio was significantly ($P < .001$) higher in the MNA group (median, 199.32; range, 17.1 to 901.6; 99% CI, 107.0 to 528.7) than in the non-MNA group (median, 0.87; range, 0.25 to 4.6; 99% CI, 0.82 to 1.26; Mann-Whitney *U* test).

Change in Serum M/N *Ratio Levels During Follow-Up*

To evaluate whether an increase in the serum *M/N* ratio can be used as an indicator of relapse, we measured serum *M/N* ratios at several points in the clinical courses of six patients with MNA (Fig 2). In three patients who were in complete remission (patients 1, 2, and 3), the serum *M/N* ratios decreased to the normal range and were consistently low. In contrast, in one patient who failed to achieve remission (patient 4), the serum *M/N* ratio did not decrease to the normal range and remained at a high level until his death. In the other patients who experienced recurrence after remission (patients 5 and 6), the serum *M/N* ratio first decreased to the normal range and then increased beyond the cutoff value by the time of diagnosis.

Effect of WBC Contamination on Serum M/N *Ratio*

We found that a high serum *M/N* ratio could be masked by the presence of WBC. The *M/N* ratio of serum from an *MYCN*-amplified patient decreased with increasing WBC contamination (Fig 3). When 200 μL of serum was contaminated with 1×10^5 of WBC, corresponding to approximately one fortieth of the WBC concentration in normal whole blood, the serum *M/N* ratio decreased below the cutoff level.

DISCUSSION

Serum markers, such as ferritin,[19] lactic dehydrogenase,[20] and neuron-specific enolase,[21] have been proposed as prognostic markers of NB, although they have shown little prognostic value. Recently, elevated levels of plasma midkine have been reported to correlate with a poor prognosis. However, the significance of this finding is controversial because plasma midkine levels are highest in stage 4S patients.[22] Therefore, a noninvasive assay of tumor-related

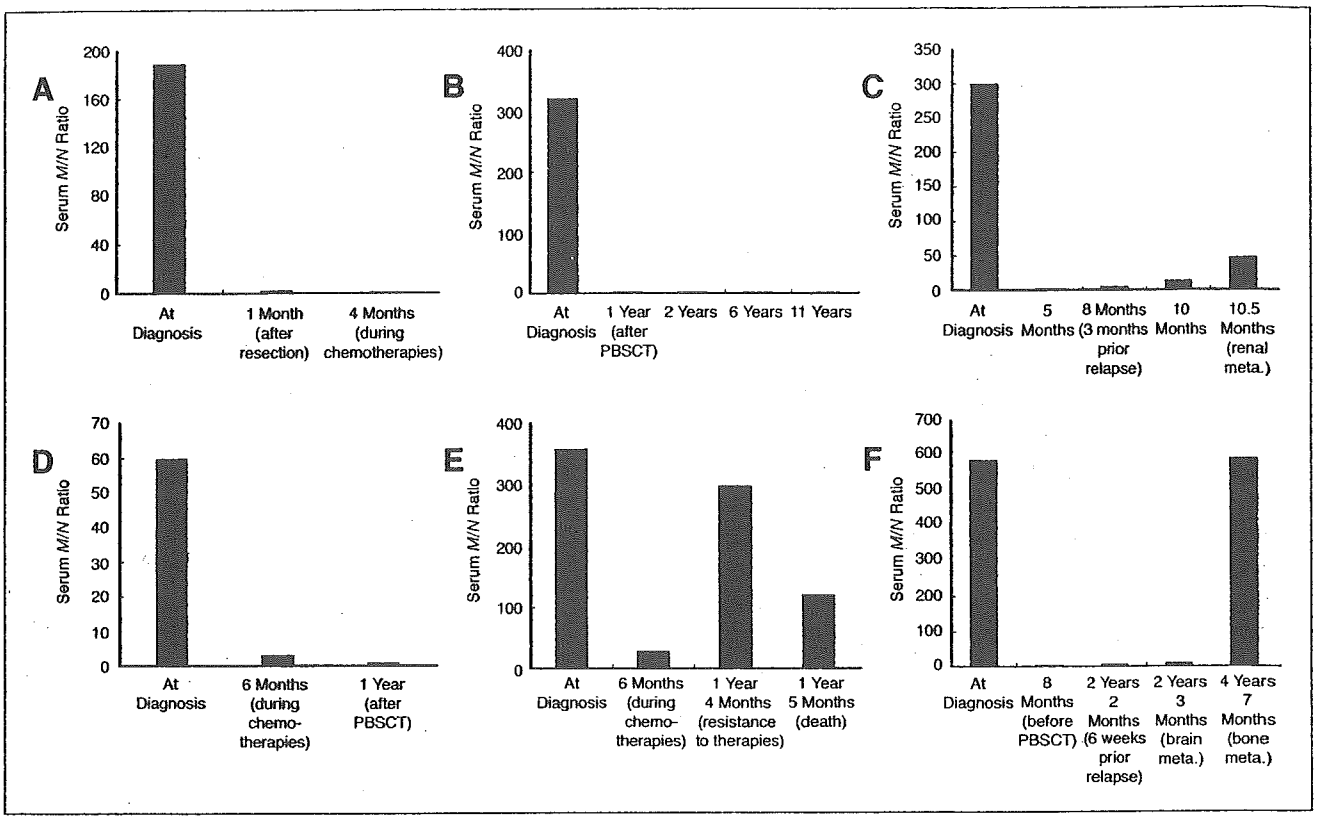

Fig 2. Changes in serum *MYCN/NAGK* (*M/N*) ratio levels of six patients with *MYCN* amplification during follow-up. PBSCT, peripheral-blood stem-cell transfusion; meta., metastasis. (A) Patient 1; (B) patient 3; (C) patient 5; (D) patient 2; (E) patient 4; (F) patient 6.

genetic aberrations using serum DNA is desirable for the assessment of prognosis and therapy stratification at the time of diagnosis. Among the tumor-related genetic aberrations detected in NB, MNA was of greatest interest to us because of its significant prognostic value.

By using DNA-based real-time quantitative PCR with a single-copy reference gene, we have demonstrated that the *M/N* ratio in serum DNA is a valuable diagnostic tool to discriminate MNA patients from non-MNA patients. The serum *M/N* ratio in the MNA group was significantly higher than the ratio in the non-MNA group, without an overlap. The highest sensitivity (100%), highest specificity (100%), highest positive predictive value (100%), and highest negative predictive value (100%) were obtained with a serum *M/N* ratio cutoff value of 10.0. Furthermore, we found an elevated level of the serum *M/N* ratio in a stage 1 patient and a stage 2B patient with MNA (188.7 and 901.6, respectively), even though the tumor was localized in these patients. This suggests that tumors could release a significant amount of genomic DNA into the systemic circulation even at an early stage. Furthermore, Sozzi et al[23] reported that the concentration of plasma DNA in 84 lung cancer patients was higher than the concentration in 43 controls, regardless of the tumor stage, and suggested that circulating DNA in peripheral blood was an early event in lung carcinogenesis.

Another clinical benefit of the serum *M/N* assay is that it could be used as a marker to monitor therapeutic efficacy and recurrence after therapies. The serum *M/N* ratio decreased to the normal range in the patients in remission but remained at a high level in the patient who failed to achieve remission. Furthermore, in two patients with recurrence after remission, the serum *M/N* ratio initially decreased to the normal range but then increased beyond the cutoff value by the time of diagnosis. The serum *M/N* ratio did not increase to the initial level as long as the metastasis was localized in the brain, but it did increase to the initial level when the patient later developed a bone metastasis (patient 6). This is noteworthy because it suggests that a brain metastasis releases genomic DNA into the systemic circulation less easily than extracranial tumors. If this is confirmed by examination of additional patients, then it is possible that tumors localized in brain could be overlooked with diagnostic assays based on serum DNA.

A possible pitfall of our serum *M/N* assay is that a high serum *M/N* ratio could be reduced by WBC contamination (Fig 3). This could be a result of dilution of tumor DNA with the WBC DNA, which would be expected to have an *M/N* ratio of 1. Therefore, the importance of removing WBCs from serum should be addressed in diagnostic assays

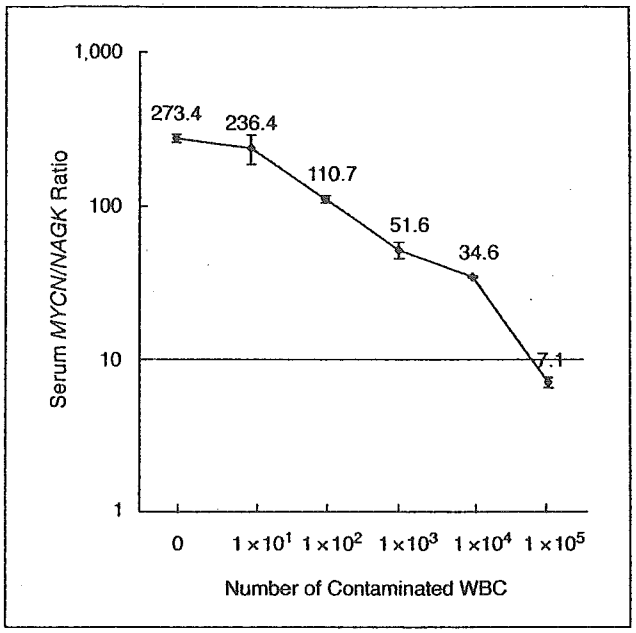

Fig 3. Influence of WBC contamination in serum samples on the serum *MYCN/NAGK* ratio. Data are presented as the mean ± standard deviation of duplicate measurements. The transverse line represents a *MYCN/NAGK* ratio cutoff value of 10.0.

that use serum DNA. For the same reason, a predominance of any nontumor DNA in serum may lower an elevated *M/N* ratio of an *MYCN*-amplified patient. However, this assay can be accurate on the premise that, in cancer patients, serum DNA predominantly consists of tumor-released DNA.[7] In addition, the use of serum DNA as a diagnostic tool in lung cancer patients has resulted in a diversity of findings, suggesting that these differences likely reflect variations in the manner in which the blood specimens were collected and handled and variations in the methods by which the assay were conducted.[24] Therefore, it is necessary to standardize the serum collection procedure to ensure that different laboratories obtain the same result with a given blood sample. An additional high-speed centrifugation step (16,000 × *g* for 5 minutes) was found to eliminate cellular contamination even after thawing of stored samples.[25] By using the appropriate centrifugation methods, we believe that WBC-free serum can be reliably achieved.

Although a large set of patients needs to be studied to verify the accuracy of this assay and to set an appropriate cutoff, our results are promising and need to be further tested. The advantages of this method are that it takes only 4 hours and much less effort than FISH and Southern blotting, which should make this assay an alternative to these other methods for determining *MYCN* status. A third advantage is that the serum *M/N* ratio seems to be a promising indicator of therapeutic efficacy and relapse in the follow-up of patients with MNA, although more patients need to be examined to confirm its reliability.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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