Detection of Single-Copy Chromosome 17q Gain in Human Neuroblastomas Using Real-Time Quantitative Polymerase Chain Reaction

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Regional genomic alterations resulting from single-copy allelic loss or gain have been well characterized in many human cancers and are often of prognostic relevance. Unbalanced gain of 17q material is common in malignant human neuroblastomas and typically results from unbalanced translocations. Unbalanced 17q gain may be an independent predictor of disease outcome, but technical difficulties with quantifying such gain using fluorescent *in situ* hybridization gives this method limited clinical applicability. We now describe a duplex genomic DNA-based quantitative polymerase chain reaction assay to determine the presence or absence of unbalanced gain of chromosome 17q in primary neuroblastoma specimens. The technique was first refined and validated in a panel of nine human neuroblastoma-derived cell lines by direct comparison with dual-color fluorescent *in situ* hybridization. Prospective blinded comparison of quantitative polymerase chain reaction and fluorescence *in situ* hybridization in 40 human neuroblastoma primary tumor samples showed a sensitivity of 96% and 100% specificity for detecting unbalanced 17q gain when a relative 17q copy number ratio of 1.3 was used to define unbalanced gain. Tumors with ratios >1.3 were highly associated with malignant tumor phenotypic features such as metastatic disease ($P <$ .0001) and tumor *MYCN* amplification ($P =$ .008). These data suggest that quantitative polymerase chain reaction determination of 17q status is feasible and highly specific in primary tumor samples. Sensitivity may be limited because of the inherent complexity of both the chromosomal rearrangements and heterogeneity of some tumor samples. Taken together, quantitative polymerase chain reaction can be used as a high-throughput screening tool for 17q aberrations, but a subset of samples may also require fluorescence *in situ* hybridization analysis in an attempt to conclusively determine 17q allelic status.

KEY WORDS: Chromosome 17, Fluorescence *in situ* hybridization, Genetics, *MYCN*, Neuroblastoma, Quantitative PCR, Unbalanced gain.

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Neuroblastoma is the most common malignant disease of infancy and the third most common pediatric cancer overall (1). It is a markedly heterogeneous disease, characterized by a wide diversity in natural history and treatment outcomes. Although patient age at diagnosis and stage of the tumor have been demonstrated to be important variables for predicting survival, it is clear that cellular and molecular features of individual primary tumors convey important additional prognostic information (2). For example, the observation that *MYCN* amplification is associated with advanced stage of disease and rapid tumor progression (3, 4) has resulted in the current standard of care of testing all diagnostic neuroblastoma samples for this genomic aberration to guide treatment planning. The Children's Oncology Group, as well as most international pediatric oncology cooperative groups, considers age at diagnosis, disease stage, Shimada histopathologic classification, DNA index, and *MYCN* amplification status in assigning patients a risk category for subsequent treatment planning (1, 5, 6). However, risk classification using these five prognostic variables

remains imprecise, and current research is focused on identifying additional tumor-specific prognostic variables that will improve the ability to properly stratify patients for treatment planning.

Unbalanced gain of 17q genomic material is a common somatically acquired abnormality in primary neuroblastomas (7–9). It has been demonstrated that these aberrations are frequently caused by unbalanced translocations that concomitantly result in loss of distal 1p, 11q, or other regions of recurrent allelic loss (7, 10–14). Unbalanced 17q gain is present at diagnosis in the more aggressive subset of neuroblastomas, suggesting that this is a somatically acquired event that contributes to a more malignant phenotype. Furthermore, the review by Bown and colleagues of 313 cases from European centers showed that unbalanced 17q gain detected by FISH was an independent marker for disease outcome (9). These data strongly suggest that risk stratification can be refined by reliable assessment of 17q allelic status in primary neuroblastoma specimens.

Dual-colored interphase FISH on tumor touch preparations appears to be a relatively sensitive and specific method for determining 17q allelic status, but this has not been tested formally. Routine FISH detection of 17q anomalies is difficult in the cooperative group setting because of the large volume of samples requiring analysis, low throughput, high cost, inadequate diagnostic material in many cases relative to what is required for high-quality touch preparations, and intratumoral heterogeneity, often making interpretation difficult. The Children's Oncology Group is interested in testing the independent prognostic impact of 17q allelic status, especially because the previous report did not include Shimada histopathology as a covariate (9), and histopathologic classification is one of the cornerstones of the Children's Oncology Group risk stratification system (1). We therefore sought to develop a relatively simple and high-throughput technique using real-time quantitative PCR (Q-PCR), and thus requiring nanogram quantities of input DNA, to screen for unbalanced 17q gain in neuroblastoma specimens.

MATERIALS AND METHODS

Samples and DNA Isolation

Nine human neuroblastoma–derived cell lines were selected from the Children's Hospital of Philadelphia neuroblastoma cell line repository. Primary tissue samples from 40 diagnostic neuroblastoma specimens were randomly selected from the Children's Hospital of Philadelphia and Children's Oncology Group Neuroblastoma Tumor Banks. Each primary tumor sample was immediately snap-frozen in liquid nitrogen on receipt in the pathology lab, according to Children's Oncology Group guidelines. Clinical and biological variables including age at diagnosis, disease stage by the International Neuroblastoma Staging System (15), DNA index (6), and Shimada pathology (5) were available for all cases, and *MYCN* amplification status was available by FISH (16) in 38/40 cases. Tumor touch preparations were made from each primary tumor and fixed in 3:1 methanol:acetic acid. DNA was extracted by anion exchange chromotography (Qiagen, Valencia, CA). The Children's Hospital of Philadelphia Institutional Review Board approved this research.

Real-Time Quantitative PCR

Q-PCR amplimers were designed to be complementary to genomic regions that showed gain (17q) or loss (17p) in all previously reported cases of unbalanced 17q gain (9, 11, 17–20). Oligonucleotide primers and probes were designed with Primer Express v1.5 (Applied Biosystems, Foster City, CA) and were complimentary to the intron 1 genomic sequence of *TP53* and the exon1–intron1 boundary within the genomic sequence of *MPO* (Table 1). The probes were differentially labeled with fluorophores VIC and FAM to allow for duplex PCR reactions, and each probe was dual labeled with TAMRA to serve as a quencher dye.

Duplex Q-PCR was performed using an ABI PRISM 7700 Sequence Detector. Initial amplification mixtures (25 μL) contained 10 ng of genomic DNA, 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 360 nM of each primer, and 100 nM of each probe. Final oligonucleotide concentrations were determined by optimization experiments in which primer concentrations were varied between 270 nM and 900 nM and probe concentrations were varied between 20 nM and 200 nM to identify combinations yielding optimal signal-to-noise and amplification efficiency. Cycling conditions were 2 minutes at 50° C and an initial denaturation step at 95° C for 10 minutes, followed by 40 cycles of 95° C for 15 seconds and 60° C for 60 seconds.

TABLE 1. Q-PCR Oligonucleotide Sequences and Fluorescence Dyes

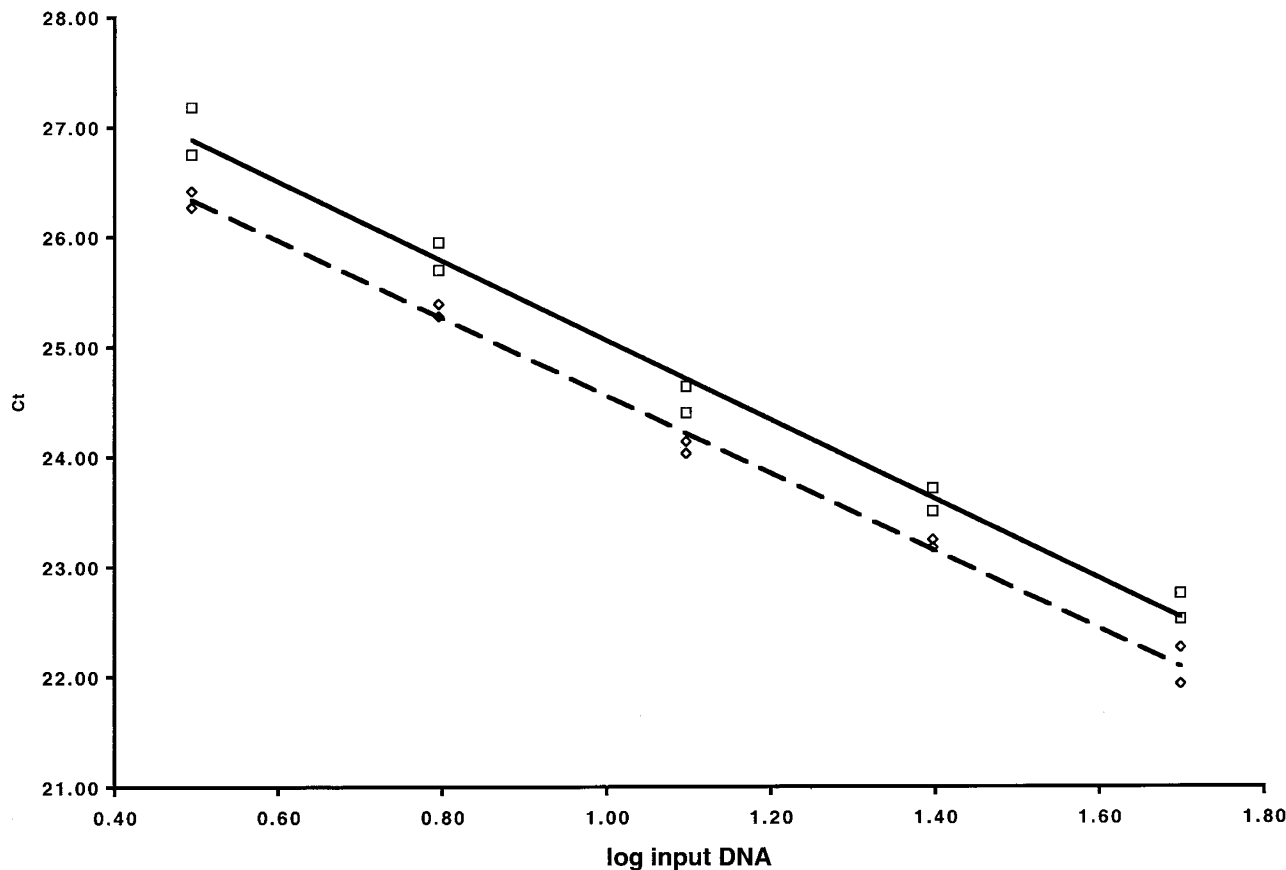|	
MPO locus at 17q23.1	
Forward Primer	AGGGCTCCTGGCCATTCT
Reverse Primer	AACCACCCCAACACACCATCT
Q-PCR Probe	*FAM*-AGGTGCTGCTCCAGGTAACAGTTCCCA-*TAMRA*
TP53 locus at 17p13.1	
Forward Primer	TGTCCTTCCTGGAGCGATCT
Reverse Primer	CAAACCCCTGGTTTAGCACTTC
Q-PCR Probe	*VIC*-CAGCCCCCGGCTCCGCTAGA-*TAMRA*

FIGURE 1. Quantitative PCR standard curves constructed with normal human genomic DNA. The amount of template input DNA is plotted against C_T value for both TP53 (***dashed***) and MPO (***solid***). All reactions were performed in duplex with both sets of primers and probes. At each serial dilution, the difference between the TP53 and MPO C_T values remains constant, indicating that the efficiencies of the reference amplification (TP53) and the target amplification (MPO) are equivalent. This relationship between the primer-probe sets validates the use of the comparative C_T method for determining the presence of 17q gain.

Initial Q-PCR experiments were performed using both the standard curve method and comparative threshold cycle ($\Delta\Delta C_T$) method (21). Standard curves were constructed using serial dilutions of normal human genomic DNA from 50 ng to 3.125 ng, and relative *MPO* and *TP53* copy numbers were extrapolated from the normal DNA standard curve using the C_T value of an individual test sample. Relative 17q copy number by the comparative C_T method was determined in both test and normal samples according to the following equation: $2^{-\Delta\Delta Ct} = (1+E)^{-\Delta Ct\ sample}/(1+E)^{-\Delta Ct\ calibrator}$, where the efficiency (E) of target and reference gene amplifications are equivalent (see validation below), $\Delta C_{Tsample}$ is the difference in C_T between the target gene and the reference gene in the test sample, and $\Delta C_{Tcalibrator}$ is the difference in threshold cycle value between the target gene and the reference gene in the normal DNA sample.

Dual-Color Interphase FISH

FISH probes were designed to overlap the Q-PCR amplimer genomic regions. DNA extracted from human bacterial artificial chromosomes clones

RP11–89D11, mapping to the *TP53* locus at 17p13.1, and RP11–506H21, mapping to the *MPO* locus at 17q23.1, were identified (Children's Hospital of Oakland Research Institute, Oakland, CA), and DNA from each clone was labeled with the Vysis Nick Translation Kit (Vysis, Inc., Downers Grove, IL). The *TP53* and *MPO* probes were labeled with Spectrum Red and Spectrum Green dUTP, respectively. Dual-color interphase FISH was performed according to the manufacturer, with minor modifications. Briefly, slides were subject to denaturation at 73° C in a solution of 70% formamide in 2× standard saline citrate and then dehydrated in ethanol. Bacterial artificial chromosomes probes were precipitated in the presence of COT-1 DNA and then denatured for 5 minutes at 73° C in LSI/WCP Hybridization Buffer (Vysis, Inc., Downers Grove, IL). Ten microliters of each probe was applied concomitantly to tumor touch preparation slides and hybridized in a moist chamber at 37° C overnight. Slides were washed with 0.3% NP-40/0.4× standard saline citrate at 73° C, and then with 0.1% NP-40/2×

standard saline citrate at room temperature, and finally they were counterstained with DAPI II.

Fluorescence signals were visualized with a Zeiss Axiophot microscope (Jena, Germany), photographed with a computer-driven Photometrics Sensys color camera, and representative images were analyzed using the Powergene system. Hybridization signals from a minimum of 75 nuclei were counted for each sample. Samples were considered to have unbalanced gain of 17q material if >50% of nuclei demonstrated one or more excess 17q signals relative to 17p signals, whereas ≤50% of nuclei with this pattern was considered no gain.

Statistical Analyses

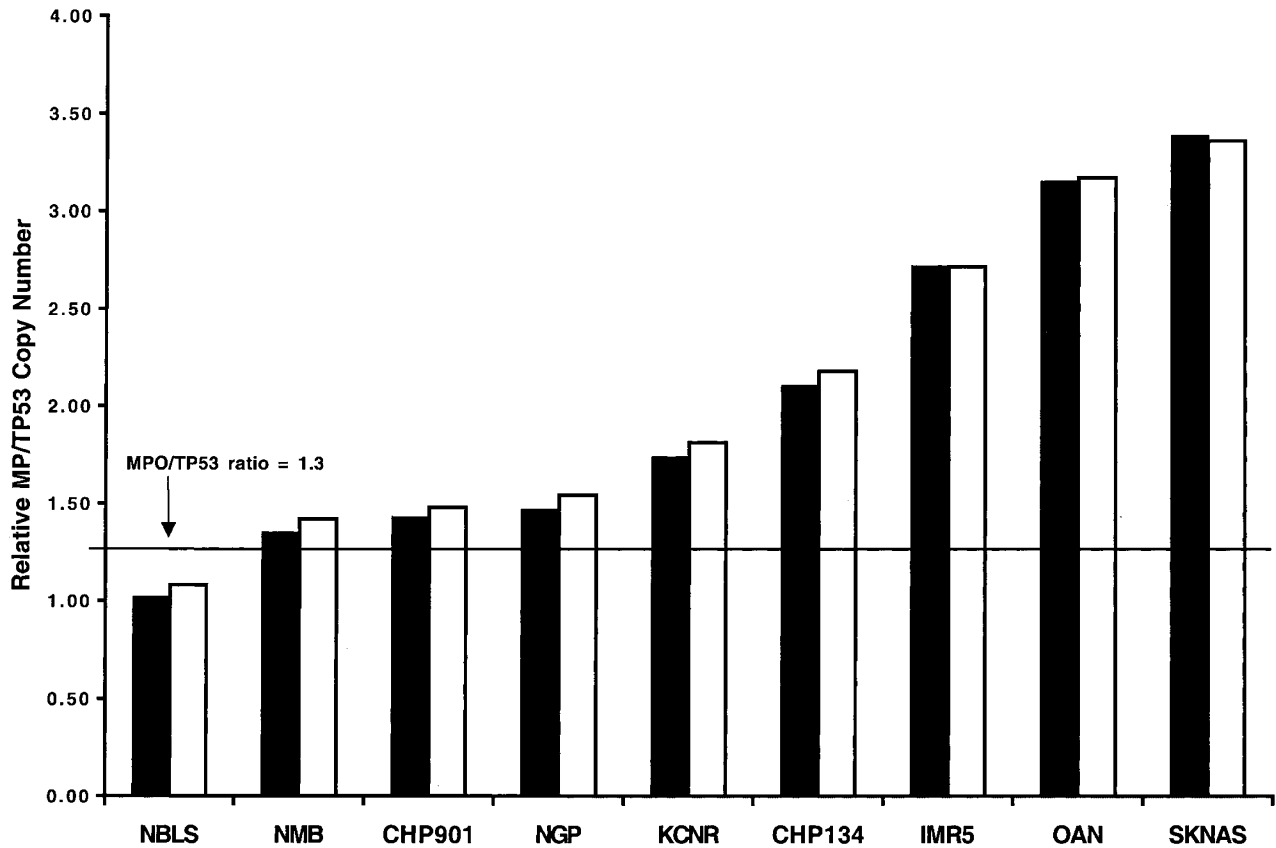Clinical and biological features were compared using the exact conditional χ^2 test.

FIGURE 2. Comparison of standard curve and comparative C_T methods for detecting 17q status in human neuroblastoma–derived cell lines. For each cell line tested, the ratio of *MPO* copy number to *TP53* copy number is shown using both the comparative C_T method (*solid*) and the standard curve method (*open*). All cell lines with unbalanced 17q gain by FISH showed relative 17q copy number ratios by Q-PCR of >1.3.

TABLE 2. Comparison of FISH and Q-PCR Results in Human Neuroblastoma-Derived Immortalized Cell Lines

Cell Line	FISH		Real-Time PCR	
	17q Status	Pattern	Std Curve Result	ΔΔCT Result
NBLS	No gain	2p2q	1.08	1.02
NMB	Gain	3p4q	1.42	1.35
CHP901	Gain	2p3q	1.48	1.43
NGP	Gain	2p3q	1.54	1.47
KCNR	Gain	2p3q	1.81	1.74
CHP134	Gain	2p4q	2.18	2.10
IMR5	Gain	2p3q	2.72	2.72
OAN	Gain	1p3q	3.17	3.15
SKNAS	Gain	1p3q	3.36	3.39

FISH results are reported by indicating the presence or absence of unbalanced 17q gain. Also shown is the predominant pattern of *TP53* (17p) and *MPO* (17q) fluorescence signals in the interphase nuclei. Real-time PCR results are reported as a ratio of *MPO* gene copy number to *TP53* gene copy number, for both the standard curve and comparative Ct methods.

RESULTS

Optimization and Validation of Q-PCR in Neuroblastoma Cell Lines

The optimal reaction concentrations determined in the serial dilution experiments were identical for both genes at 360 nM for each primer and 100 nM for each probe (data not shown). Duplex Q-PCR was then performed on serial dilutions of normal genomic DNA. PCR reaction kinetics were similarly efficient for each gene (R ≥ 0.99), suggesting that a comparative C_T ($\Delta\Delta C_T$) method was an acceptable strategy for relative quantification of 17q copy number (Fig. 1) (21). Both the standard curve and the $\Delta\Delta C_T$ methods were used to determine 17q status in nine neuroblastoma cell lines. Results using both methods were essentially identical (Fig. 2; Table 2). The $\Delta\Delta C_T$ method was therefore used for further protocol development because it allows for higher sample throughput by obviating the need for serial dilutions in each experiment.

We next compared Q-PCR results with copy number determination by dual color interphase FISH. In our sample set of nine cell lines, NBLS was the only example of a normal 17p-17q allelic ratio by FISH (Fig. 2; Table 2), and this is not surprising because of the high correlation between 17q gain and the high-risk phenotype that is required to establish cell lines. This was the only sample that showed a relative 17q copy number determination of <1.3. The remaining samples showed a variety of copy number patterns by FISH with either a one- or two-copy difference between 17q and 17p. Relative 17q copy numbers ranged from 1.35–3.39, and these values in general correlated with the degree of discrepancy in 17p and 17q allelic copy number by FISH (Table 2).

Prospective Evaluation of Q-PCR in Neuroblastoma Primary Tumors

Q-PCR and dual-color interphase FISH were performed on 40 primary human neuroblastoma samples by individual investigators who were blinded to the results for the alternate technique. All samples were classified as unbalanced 17q gain present or absent by dual-colored FISH and Q-PCR (Fig. 3; Table 3). All samples could be categorized by FISH, although many samples had complex patterns of 17q rearrangements with multiple distinct fluorescence signal patterns. Table 3 shows the predominant pattern observed. Q-PCR results ranged from 0.69–2.84 and correlated with the relative 17q copy number determination by FISH (Fig. 4). We explored different relative 17q copy number values for the binary determination of presence or absence of
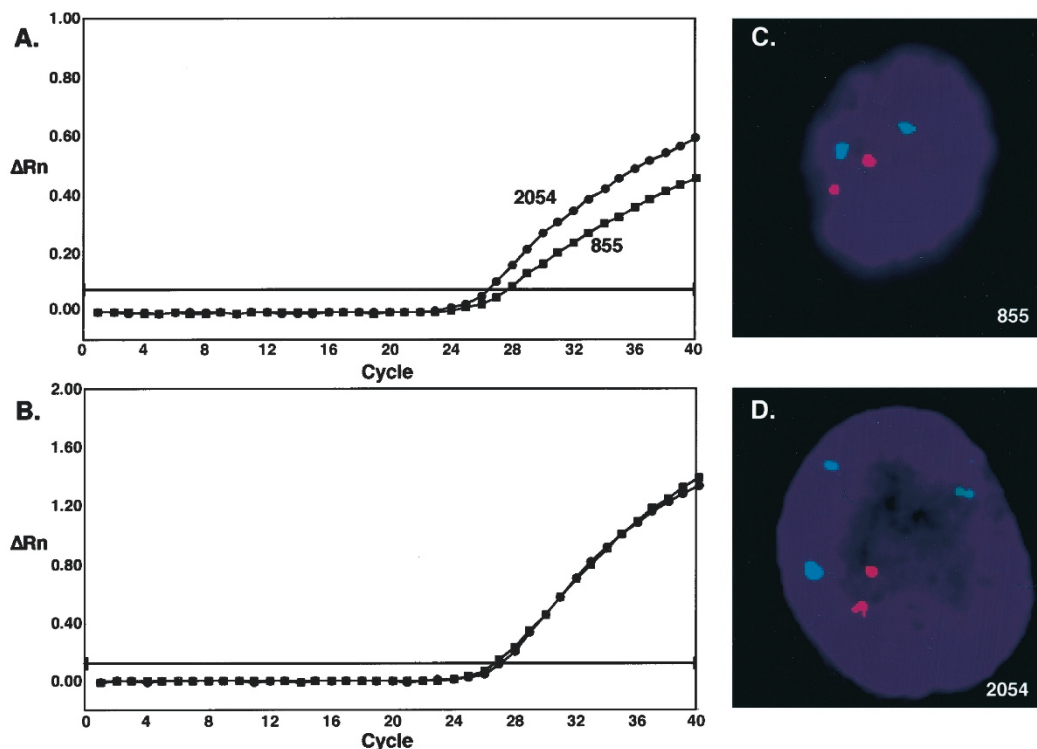
FIGURE 3. Correlation of Q-PCR and FISH in two primary tumors. Amplification plots of *MPO* (**A**) and *TP53* (**B**) fluorescent signal *versus* cycle number for primary neuroblastomas 855 and 2054. The amplification plots are essentially identical for *TP53*, but tumor 2054 has a lower C_T value for MPO. Using the ΔΔCT method, these results correspond to a 17q-17p ratio of 1.9 for tumor 2054 and of 1.0 for tumor 855. These data are confirmed by dual-colored interphase FISH using probes to *MPO* (**green**) and TP53 (**red**). Shown are representative single nuclei showing no gain of 17q material for 855 (**C**) and unbalanced 17q gain for 2054 (**D**).

unbalanced 17q gain (Table 4). Using a relative 17q copy number of 1.4 as a cut point showed 100% specificity (no false positives), but 6 false negatives (74% sensitivity). Relaxing the stringency to 1.3 again showed 100% specificity with only 1 false negative (96% sensitivity). Specificity decreased to 76% when the relative 17q copy number cut point was further relaxed to 1.2, but the sensitivity was 100%.

Primary neuroblastomas with relative 17q copy number values of >1.3 were significantly more likely to be from patients with metastatic disease (Table 3; $P < .0001$). Concomitant *MYCN* amplification occurred exclusively in this group of tumors with high relative 17q copy number values ($P = .008$). These tumors also tended to be from patients older than 1 y of age ($P = .07$).

DISCUSSION

Neuroblastoma is a clinically heterogeneous disease, and a large number of patients have an outstanding prognosis that can be predicted based on analysis of clinical and tumor biological variables. However, the majority of neuroblastoma cases show hematogenous metastases at diagnosis that is often refractory to treatment (1). Understanding the molecular features that discriminate the unfavorable from the favorable subsets of neuroblastoma has been a focus of intense research in recent years (reviewed in 2). To date, this research has identified a relatively large panel of somatically acquired genomic alterations that correlate with tumor natural history, including response to treatment. Neuroblastoma has served as a paradigm for the clinical

TABLE 3. Comparison of FISH and Q-PCR Results in Human Neuroblastoma-Primary Tumor Samples

Sample	Age at Diagnosis (days)	Stage	MYCN[1]	FISH 17q Status[2]	FISH Predominant Pattern (%)	Q-PCR ΔΔCT Ratio
305	736	2b	Not Amp	No UG	4p4q (30%)	0.69
337	93	1	Not Amp	No UG	2p2q (13.6%)	0.90
401	1846	3	Not Amp	No UG	4q4p (56%)	0.95
249	173	3	Not Amp	No UG	2p2q (46%)	0.97
314	218	2b	Not Amp	No UG	2p2q (13.4%)	0.97
343	38	1	Not Amp	No UG	3p3q (19.3%)	1.00
855	1747	2b	Not Amp	No UG	2p2q (92%)	1.00
344	2980	3	Not Amp	No UG	3p3q (36%)	1.04
3	417	3	Not Amp	No UG	2p2q (91%)	1.07
756	5	4s	Not Amp	No UG	3p3q (48%)	1.07
329	70	4	Not Amp	No UG	4p4q (28.7%)	1.09
327	6	2b	Not Amp	No UG	4p4q (8.9%)	1.11
1995	709	2b	Not Amp	No UG	4p4q (73%)	1.17
796	377	3	Not Known	UG	2p3q (67%)	1.23
351	1852	4	Not Amp	No UG	4p4q (16.7%)	1.23
308	69	1	Not Amp	No UG	6p6q (19.3%)	1.25
304	421	2b	Not Amp	No UG	4p4q (20.8%)	1.27
2087	721	1	Not Amp	No UG	2p2q (53%)	1.28
5	790	4	Amp	UG	3p4q (79%)	1.32
320	514	4	Amp	UG	3p3q (19.8%)	1.33
393	280	4	Amp	UG	2p4q (25%)	1.36
334	907	4	Not Amp	UG	2p3q (26.7%)	1.38
312	2269	4	Not Amp	UG	6p7q (11.4%)	1.39
923	276	4	Not Amp	UG	2p3q (72%)	1.68
1652	330	4	Not Amp	UG	2p3q (70%)	1.70
340	916	4	Not Amp	UG	2p4q (34.7%)	1.75
357	100	4s	Not Amp	UG	3p3q (12.9%)[3]	1.76
1673	485	4	Not Amp	UG	2p4q (49%)	1.81
2054	1131	4	Amp	UG	2p3q (67%)	1.90
306	659	4	Not Amp	UG	2p3q (20.2%)	1.92
1554	1525	4	Not Amp	UG	2p5q (56%)	1.94
1848	1555	4	Not Amp	UG	2p4q (40%)	1.96
333	1189	4	Not Amp	UG	3p7q (8.7%)	2.06
319	594	4	Not Amp	UG	2p4q (58.5%)	2.18
1994	1422	4	Amp	UG	2p4q (66%)	2.49
341	1002	4	Not Amp	UG	2p5q (50%)	2.51
1749	841	4	Not Known	UG	3p6q (58%)	2.63
317	895	4	Amp	UG	1p3q (37.5%)	2.74
140	935	4	Amp	UG	1p3q (72%)	2.78
347	1070	4	Not Amp	UG	2p5q (22.2%)	2.84

FISH results include the presence or absence of unbalanced gain of chromosome 17q material as well as the dominant hybridization. These are compared with the ratio of MPO (17q) gene dosage to TP53 (17p) gene dosage, as measured by Q-PCR.

[1] Amp = amplified; not amp = not amplified.

[2] UG = unbalanced gain of chromosome 17q; no UG = no gain or balanced gain of both 17p and 17q.

[3] Sample 357 contained a heterogeneous sample of nuclei with a highly variable pattern of 17p and 17q FISH signals. Although 59% of nuclei showed unbalanced gain of 17q material, the single most observed pattern of signals was 3p3q.
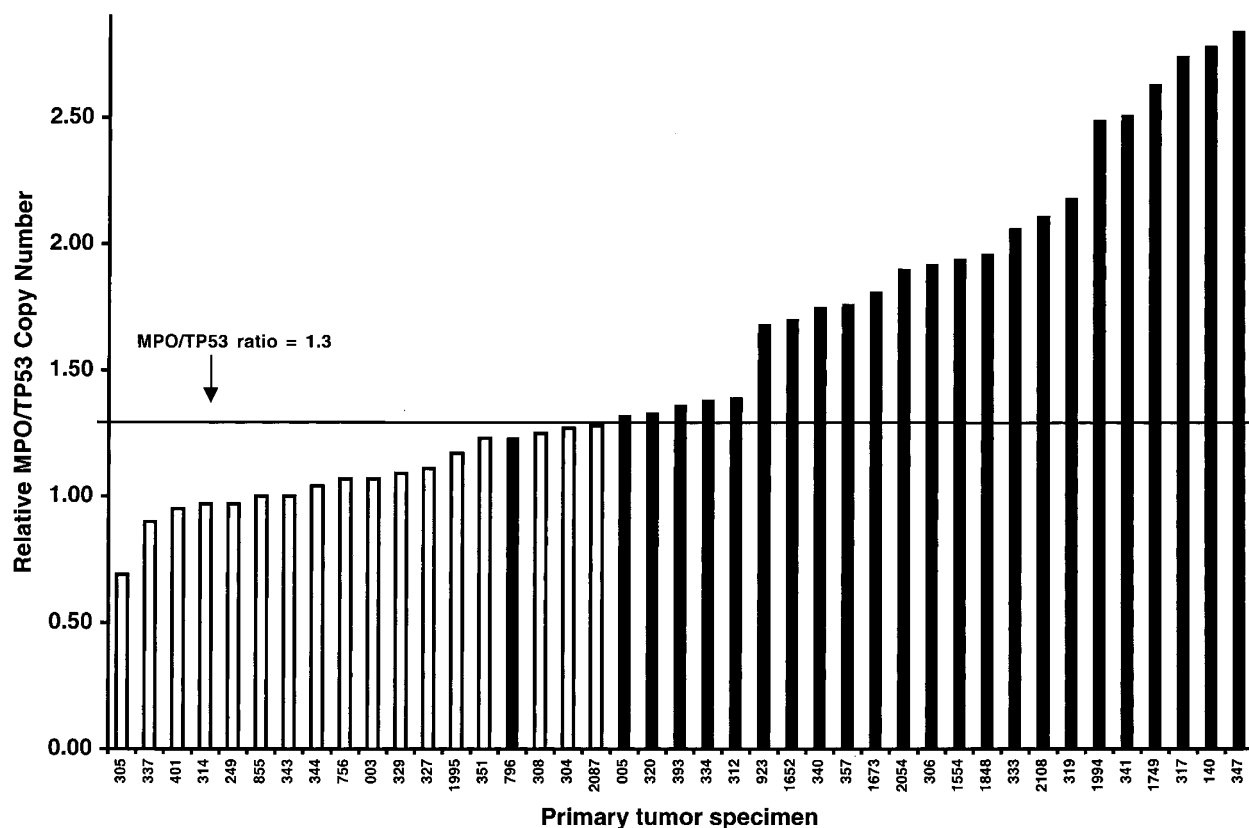
FIGURE 4. Chromosome 17 status of primary human neuroblastoma specimens, as determined by Q-PCR. Tumor samples with 17q gain as determined by FISH are represented as ***open bars,*** whereas those with 17q gain determined by FISH are seen as ***filled bars.*** All tumor samples with unbalanced 17q gain by FISH showed relative 17q copy number ratios by Q-PCR of >1.3, with the exception of sample 796.

utility of such information, as *MYCN* oncogene copy number status is used worldwide for risk stratification. Ongoing research is aimed at refining our current risk stratification algorithm by introducing additional tumor-specific prognostic markers that improve our ability to predict tumor behavior.

The current literature supports a strong association between unbalanced gain of chromosome 17q material and an adverse clinical outcome (8, 9, 18, 22–24). In addition, 17q copy number status may be an independent predictor of outcome even when patient age at diagnosis, disease stage, and *MYCN* amplification status are considered (9). The major obstacle for incorporating this marker into routine molecular diagnostics is the technical difficulty that is associated with detecting relatively subtle copy number alterations by FISH. This technique is labor intensive, costly, and of low throughput. Therefore, a reliable PCR-based assay that can be used for the high-throughput screening of 17q aberrations in diagnostic neuroblastoma samples would allow for definitive clinical correlative studies aimed at determining the clinical utility of this potential prognostic marker.

Real-time quantitative PCR has been adapted to detect high-level genomic amplification in solid tumor samples, including neuroblastoma (25). How-

ever, quantitative assessment of relatively subtle copy number gain in clinical samples has not been reported. The assay described here was designed to provide a relative 17q-17p copy number ratio because unbalanced gain of 17q material has been shown to be prognostically relevant (9, 10, 14, 26, 27). Primer and probe sets were designed based on previous mapping and clinical correlative studies using FISH probes to detect relative abundance of *MPO* (17q23.1) compared with *TP53* (17p13.1) (9, 11). The assay was optimized to allow for use of the comparative Ct method for determining gene copy number, allowing for high throughput by obviating the need for an experiment-specific serial dilution

TABLE 4. Comparison of Q-PCR Data with FISH Analysis for Relative 17q Copy Number Using Different Q-PCR Cut Points

	FISH	
	Gain	No Gain
Q-PCR		
≥1.2	23	4
<1.2	0	13
≥1.3	22	0
<1.3	1	17
≥1.4	17	0
<1.4	6	17

to allow for construction of a standard curve. Data from established cell lines showed the technique to correlate well with interphase FISH data, thus validating the experimental strategy in the artificial setting of uncontaminated cellular material.

For this assay to be of clinical utility, it must be both a sensitive and specific method for detecting unbalanced 17q gain in clinical samples. Neuroblastomas show varying degrees of "contaminating" normal tissues, including vascular and supporting stroma and infiltrating Schwann cells (28). Our data suggest that despite intratumoral cellular heterogeneity, Q-PCR can unequivocally determine 17q allelic status in the majority of clinical samples. In the relatively small sample set studied here, a relative 17q copy number value of 1.3 provided optimal discrimination between tumors with and without unbalanced 17q gain. However, it is also apparent that Q-PCR will provide continuous data across a large sample set, suggesting that this PCR-based technique will not be capable of providing 100% sensitivity and specificity. This should not be surprising, as a significant subset of samples often cannot be reliably classified for 17q status using FISH. Therefore, we plan to score samples with relative 17q copy number values of 1.2–1.4 in future studies as indeterminate by Q-PCR. Each of these samples will then also be examined by dual-color interphase FISH in an attempt to unequivocally assign 17q allelic status for all specimens. Ongoing studies in a much larger number of samples may determine a more precise relative 17q copy number cutoff value and range for samples requiring FISH evaluation.

Q-PCR as described here provides for high throughput screening of relative 17q copy number in primary neuroblastoma specimens. Further studies may refine this assay toward near-perfect sensitivity and specificity, but we expect that there will always be a subset of tumors that should also be studied by FISH if unequivocal determination of 17q allelic status is the goal. Even without further refinement, this assay will allow for highly specific 17q status assignment for the vast majority of neuroblastomas, thus streamlining integration of this molecular marker into clinical correlative studies. Results from prospective evaluation of the clinical significance of 17q aberrations in large numbers of human neuroblastomas may lead to refinements in risk stratification algorithms and thus to more precise allocation to therapeutic protocols.

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