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Segmental Chromosomal Aberrations in Localized Neuroblastoma Can be Detected in Formalin-Fixed Paraffin-Embedded Tissue Samples and Are Associated With Recurrence

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Abstract

Background—Array comparative genomic hybridization (CGH) analyses of frozen tumors have shown strong associations between the pattern of chromosomal aberrations and outcome in patients with advanced-stage neuroblastoma. New platforms for analyzing chromosomal aberrations using formalin-fixed paraffin-embedded (FFPE) tissue have recently been developed. We sought to determine whether chromosomal microarray analysis (CMA) using FFPE tumors is feasible and if segmental chromosomal aberrations were prognostic of recurrence in localized neuroblastoma.

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Methods—Patients with *MYCN* nonamplified International Neuroblastoma Staging System stage 1 and 2 disease who recurred were identified. CMA was performed with diagnostic FFPE samples using OncoScan™ FFPE Express 2.0. The prognostic significance of chromosomal pattern was validated in 105 patients with available CGH results.

Results—In 26 evaluable patients, 11 recurred locally, nine had metastatic relapse, and six remained progression free >3 years from diagnosis. No chromosomal aberrations were identified in four tumors. Numerical chromosomal aberrations (NCAs) without segmental chromosomal aberration (SCA) were identified in 11 patients: six progressed locally, two had metastatic progression and 3 remained progression-free. Eleven patients had SCAs: four progressed locally, six developed metastatic progression and one remained progression-free. Five or more SCAs were only detected in tumors from patients who developed metastases ($P = 0.0004$). In the validation cohort, SCAs were associated with inferior event-free survival (EFS) compared to NCA (5-year EFS $68\% \pm 8.3\%$ vs. $91\% \pm 3.6\%$, respectively; $P = 0.0083$).

Conclusions—It is feasible to evaluate chromosomal aberrations using FFPE neuroblastoma tissue. SCA is associated with inferior EFS in localized neuroblastoma patients, and multiple SCAs may be predictive of metastatic relapse.

Keywords

low-risk neuroblastoma; metastatic relapse; segmental chromosomal aberrations

BACKGROUND

Neuroblastoma is the most common extracranial solid tumor of childhood. While outcome for high-risk patients remains poor, more than 90% of patients with low-risk, localized disease (INSS stages 1 and 2) are cured following surgery alone or surgery and moderate-dose chemotherapy.[1–4] The vast majority of relapses that occur in this cohort are in the same site as the primary tumor, although rare patients will develop metastatic relapse.[2,4] Most patients with local tumor progression will be successfully salvaged with additional treatment. In contrast, low-risk patients who relapse in metastatic sites are more difficult to cure. This subset of patients may benefit from more intensive therapy at the time of diagnosis, when the tumor burden is low. However, it is currently not possible to distinguish the rare patients with *MYCN* nonamplified localized tumors who will progress or relapse in distant sites from those who will remain disease free.[3]

Previous studies using array comparative genomic hybridization (aCGH) and frozen tumor samples have demonstrated that the presence of numerical (or whole) chromosomal aberrations (NCAs) and segmental chromosomal aberrations (SCAs) is prognostic of outcome in children with neuroblastoma.[5–8] NCAs are more common in infant tumors and are associated with favorable outcomes. In contrast, SCAs are more prevalent in patients with high-risk neuroblastoma. Further, the presence of SCA compared to NCA is associated with inferior survival for infants with advanced-stage disease and children with unresectable locoregional tumors.[5–8] The number of segmental aberrations has also been shown to correlate with outcome, and children with tumors with >7 chromosomal aberrations have a dismal outcome.[6] However, the prognostic value of chromosomal aberrations in patients

with resectable localized tumors that lack *MYCN* amplification (INSS stage 1 and 2) remains less clear.

Previously, we evaluated the pattern of chromosomal aberrations in two patients diagnosed with INSS stage 1 or 2 *MYCN* nonamplified neuroblastoma who subsequently relapsed in metastatic sites.[9] Using an array platform that was specifically optimized for formalin-fixed paraffin-embedded (FFPE) samples, we detected more than seven SCAs in both diagnostic FFPE tumor samples. Amplified domains within chromosome 12 were also identified in one patient's tumor sample, in a region that has previously been reported to be associated with aggressive disease.[10] These two cases demonstrated that chromosomal aberrations could be analyzed in FFPE neuroblastoma tumor samples, and suggested that the presence of multiple SCAs may be predictive of metastatic relapse in patients initially presenting with low-risk, localized tumors. To further investigate the feasibility of analyzing chromosomal aberrations in FFPE neuroblastoma tumor samples, and to determine whether the presence of SCA in stage 1 and 2 neuroblastoma is prognostic of recurrence and/or the pattern of disease progression (local versus metastatic), we analyzed diagnostic FFPE tumors in a cohort of patients with localized *MYCN* nonamplified tumors, enriched for patients who had recurred following initial treatment. To validate that prognostic value of chromosomal pattern, we analyzed survival in a cohort of 105 previously reported patients with localized, *MYCN* nonamplified neuroblastomas with available frozen tumor aCGH results.[6,11]

METHODS

Patients and Tumor Samples

Neuroblastoma patients from Comer Children's Hospital at the University of Chicago, Children's Hospital of Philadelphia, Hospital for Sick Children (Toronto), Children's Hospitals and Clinics of Minnesota, and the Ann and Robert H. Lurie Children's Hospital at Northwestern University diagnosed between 1989 and 2013 with localized, *MYCN* non-amplified stages 1 or 2 disease based on Children's Oncology Group (COG) criteria [12] who recurred were identified. Additional stage 1 and 2 patients diagnosed at Comer Children's Hospital between 2001 and 2008 who remained progression free for more than 3 years from diagnosis were also included in the study cohort. Medical records were abstracted at each institution and clinical data including age of diagnosis, sex, stage, *MYCN* status, ploidy, histology, and outcome were collected. The patients were staged according to the International Neuroblastoma Staging System and tumor histology was defined as favorable or unfavorable using the International Neuroblastoma Pathologic Classification System.[13,14] Four to ten FFPE scrolls of diagnostic tumor tissue were sent to the University of Chicago. This study was approved by the Institutional Review Board at the University of Chicago and at each of the collaborating institutions.

A separate survival analysis using aCGH data derived from frozen (as opposed to FFPE) neuroblastoma tumors was performed. Only the subset of patients with resectable neuroblastoma tumors that lacked *MYCN* amplification was included.[6,11] Patients were diagnosed between 1987 and 2006, and frozen tumor samples from these patients had previously been analyzed by aCGH.

DNA Isolation and Genomic Profile

DNA was isolated from FFPE tumor samples using the Qiagen PureGene DNA extraction kit reagents (Qiagen, Hilden, Germany), following the manufacturer's guidelines. Tumor content was confirmed either by review of hematoxylin and eosin stained tumor sections by the local pathologists at each of the institutions or review of the pathology report at the University of Chicago. Chromosomal microarray analysis (CMA) was performed at The University of Chicago using the OncoScan™ FFPE Express 2.0 System (Affymetrix, Santa Clara, CA). Data analysis was performed using the Oncoscan Nexus Express Software (Biodiscovery, Hawthorne, CA) and whole chromosome gains and losses, copy number aberrations (deletions and duplications), and loss of heterozygosity events were determined. NCAs were defined as gains or losses of whole chromosomes relative to the established ploidy level, while SCAs were defined as copy number gains or losses affecting one chromosome arm or a segment within a chromosome arm. To characterize a change as a SCA, at least 100 contiguous oligonucleotide probes were required to exhibit a different copy number status compared to the rest of the chromosome. For each sample, a chromosomal pattern was assigned: "NCA" for samples with only numerical aberrations, "SCA" for samples with segmental aberrations with or without numerical aberrations, and "Silent" for samples without numerical or segmental aberrations. The number and location of segmental duplications and losses in each tumor were determined.

Quality Control

Based on manufacturer's recommendations, two quality control (QC) metrics, automatically generated by the array analysis software, were used to evaluate the quality of the data: Median of the Absolute Values of all Pairwise Differences (MAPD), which is a global measure of the variation of all microarray probes across the genome, and Single Nucleotide Polymorphism Quality Control (SNPQC), which is a measure of how well the alleles with different genotypes are resolved in the microarray data. The target value for MAPD was 0.30, while the target value for SNPQC was 26. Because the cases were rare and no additional material was available, the arrays that did not meet the QC metrics were not automatically rejected; such arrays were carefully reviewed and accepted for further analysis if the copy number data could be clearly interpreted.

Statistical Analysis

The Fisher exact test [15] was performed to evaluate associations between segmental chromosomal arrangements and clinical groups defined as (i) no evidence of disease progression, (ii) local recurrence, and (iii) metastatic relapse. Survival curves were estimated according to the Kaplan–Meier method [16] with one-sided log-rank tests to compare cohorts according to the presence or absence of SCA.

RESULTS

Patient Cohort

The clinical and tumor characteristics are summarized in Table I for the patients who had CMA performed using FFPE tumor samples, Figure 1 provides representative visualizations

of NCA and SCA patterns. The analytic cohort consisted of 26 patients diagnosed with *MYCN* nonamplified stage 1 or 2 disease. Fifteen patients were less than 18 months old at the time of diagnosis and 10 patients had thoracic primary tumors. Fourteen patients had favorable histology tumors. Initial treatment consisted of observation ($n = 1$), surgery alone ($n = 21$), or surgery and moderate-dose chemotherapy ($n = 4$). Eleven patients recurred at the site of their primary tumors without evidence of metastatic disease at a median of 11 months from diagnosis (range 3–54 months), nine relapsed at metastatic sites with or without a local recurrence at a median of 5 months (range 3–11 months), and six remain progression free more than 3 years from diagnosis (range 20–38 months). Time to relapse was not available for two patients with metastatic relapse.

To further evaluate the prognostic value of SCA in localized neuroblastoma, we analyzed an additional 105 patients with resectable tumors treated with surgery alone who were included in a previously reported cohort of 394 patients enrolled on European studies (Table II).[6,11] Sixteen of the 105 recurred. Ten patients developed local recurrence, information regarding the metastatic workup is not available on four of these patients. Three patients experienced metastatic relapse, and an additional three patients were known to have a combined metastatic and local relapse.

CMA

Tumors from a total of 27 patients were analyzed and CMA data were evaluable in 26 tumors. One patient was inevaluable due to insufficient tumor cell content and was not included in the analysis. In four tumors, no chromosomal aberrations were identified despite a sufficient tumor cell content in the sample including a tumor from one patient with a known family history of neuroblastoma. Chromosomal aberrations were detected in four of the six patients in this cohort who remained progression free. A NCA genomic profile was observed in three tumors and a single SCA was present in one tumor. In the tumors from the 11 patients who developed a localized recurrence, NCAs were identified in six, SCAs were identified in four, and one had a tumor that was silent for chromosomal aberrations. In the tumors from the nine patients who developed relapse in metastatic sites, two had NCA and six had SCA. One patient who developed disseminated relapsed disease had a tumor with no detectable chromosomal aberrations. Recurrent chromosome imbalances previously observed in neuroblastoma (gain of 2p, 17q, and loss of 1p, 3p, 4p, 11q) were observed in tumors with SCA (Table III). Six of the nine patients who relapsed in metastatic sites had tumors with both 17q gain and 11q deletions. The number of segmental aberrations also correlated with metastatic relapse in this cohort. Patients with tumors that contained five or more SCA at the time of diagnosis were significantly more likely to develop metastatic relapse ($P = 0.0004$).

In the validation cohort, the presence of SCA was strongly associated with recurrence ($P = 0.0033$). SCAs ($n = 2$ –15) were detected in 10 of the 16 (62.5%) patients who developed a local recurrence. Of the 89 patients without a recurrence, SCAs ($n = 1$ –7) were identified in 23 (25.8%) tumors.

Five-year event-free survival (EFS) for patients with NCA was $91\% \pm 3.6\%$ versus $68\% \pm 8.3\%$ for patients with tumors with SCA ($P = 0.0083$, Fig. 2A). The presence of a single

SCA was not associated with disease recurrence (six patients, 5-year EFS 100%, Fig. 2B). Two patients died, and SCAs (n = 2 and 9, respectively) were detected in the tumors from these patients. Although a trend toward inferior survival in patients with SCA was observed, a statistically significant difference in overall survival (OS) was not detected between the cohorts with NCA versus SCA. Ten-year OS was 100% for patients with NCA versus 88.1% for patients with SCA (hazard ratio 13.7 [95% confidence interval [CI] 0.78–240]; $P = 0.07$). SCA was present in six of 10 patients who experienced a local recurrence and in four of six patients who relapsed in metastatic sites. In this cohort, the number of SCA was not associated with the pattern of disease progression.

DISCUSSION

In this study, we analyzed the feasibility of detecting chromosome gains and losses in FFPE neuroblastoma tumor samples using the OncoScan™ FFPE Express 2.0 System (Affymetrix, Santa Clara, CA), and we investigated the clinical relevance of chromosomal aberrations in patients with localized disease. Because of the low incidence of recurrence in patients with stages 1 and 2 tumors, we specifically sought diagnostic localized tumors from patients who had developed recurrent disease. We show that chromosome aberrations can be detected in FFPE tumor samples. In our study cohort, chromosomal aberrations were common, with NCA and/or SCA detected in 22 of the 26 tumors. Similar to previous studies conducted in patients with advanced-stage disease,[5–8,11] in this cohort the presence of SCA in localized tumors was associated with clinically aggressive disease. Of the 11 patients with SCA, 10 developed recurrence: four had local progression and six relapsed in metastatic sites. We also found that multiple SCAs were seen in tumors with the most aggressive behavior, in agreement with a previous aCGH study.[6] One to three SCAs were identified in the tumors from patients who recurred locally, whereas 5–11 SCAs were observed in the tumors from patients who developed metastatic relapse. All six patients who had five or more SCAs in their diagnostic tumors developed metastatic relapse.[6] Given our selection bias of our study cohort toward relapsed patients, we sought to validate the unfavorable prognostic significance of SCA in patients with localized neuroblastoma in an independent cohort of 105 European patients with localized disease treated with surgery alone. In this cohort, SCA was significantly associated with inferior EFS compared to patients with NCA. SCAs were detected in tumors from 62.5% of patients who developed recurrent disease, compared to 25.8% of patients who remained recurrence free after surgery. Interestingly, the presence of a single SCA was not associated with disease recurrence (six patients, 5-year EFS 100%, Fig. 2B). In patients with SCA, gain of chromosome 17q was the most frequently observed chromosomal aberration (23/33, 69.7%); seven of 33 (21.2%) patients had deletions of chromosome 11q. Although the pattern of disease progression (local vs. metastatic) was not associated with the number of SCA in the European validation cohort, multiple SCAs were detected in the tumors from the two patients who died.

Although our study was limited by the small number of FFPE tumors evaluated and the retrospective nature of the analysis, we demonstrate that it is feasible to detect NCA and SCA in FFPE tumor samples using CMA. We also show that the presence of SCA in localized tumors is associated with inferior EFS compared to NCA, and that multiple SCAs may identify patients with clinically aggressive tumors who may benefit from more intensive

treatments at diagnosis. Further investigation of the clinical significance of SCA in a prospective clinical study is warranted.

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Fig. 1.

X axis represents chromosome location. Y axis represents the $\log_2$ of the ratio of signal intensities between the tumor (test sample) and normal controls. **(A)** CMA results from a diagnostic tumor from a patient diagnosed with a localized tumor who developed local progression disease showing NCA of chromosomes 1-3, 5-7, 11-13, 15-20, and 22. **(B)** CMA results from a diagnostic tumor from a patient who presented with a localized tumor and relapsed with metastatic disease showing SCA of chromosomes 1, 2, and 16 and NCA of chromosomes 3-7, 9-10, 12, 14-15, 17, and 19-21.

Fig. 2.

Five-year event-free survival (EFS) of the validation cohort by chromosomal pattern. **(A)** Five-year EFS of patients with NCA compared to SCA. **(B)** Five-year EFS of patients with NCA compared to those with one SCA versus greater than one SCA.

TABLE I

Clinical and Tumor Characteristics of the Discovery Cohort (n = 26)

Characteristic	Number (%)	> 1 SCA (%) ^a
Age		
18 months	15 (range 0–15, median 2.2 months) (57.7)	2 (13.3)
>18 months	9 (range 20–88, median 41 months) (34.6)	6 (66.7)
Unknown	2 (7.7)	1 (50)
Gender		
Male	15 (57.7)	6 (40)
Female	9 (38.5)	2 (22.2)
Unknown	2 (7.7)	1 (50)
INSS		
1	13 (50)	2 (15.4)
2a	3 (11.5)	0 (0)
2b	7 (26.9)	6 (85.7)
Localized, but unknown stage	3 (19.2)	1 (33.3)
Primary tumor location		
Adrenal	7 (26.9)	2 (28.6)
Neck	2 (7.7)	1 (50)
Thorax	10 (38.5)	4 (40)
Pelvic	3 (11.5)	0 (0)
Abdominal/retroperitoneal	2 (7.7)	1 (50)
Unknown	2 (7.7)	1 (50)
Histology		
Favorable	15 (57.7)	2 (13.3)
Unfavorable	8 (30.8)	6 (75)
Unknown	3 (11.5)	1 (33.3)
Ploidy		
Hyperdiploid	11 (42.3)	2 (18.2)
Diploid	5 (19.2)	3 (60)
Unknown	10 (50)	4 (40)
Initial treatment		
Observation	1 (3.8)	1 (100)
Surgery only	19 (73.1)	4 (21)
Standard dose chemotherapy + surgery	4 (15.4)	3 (75)
Unknown	2 (7.7)	1 (50)
Relapse		
None	6	0 (0)

Characteristic	Number (%)	> 1 SCA (%) ^a
Local	11 (42.3)	3 (27.3)
Metastatic	9	6 (66.7)

^aPercent of the number with this characteristic.

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TABLE II

Clinical Characteristics of the Validation Cohort (n = 105)

Characteristic	Number (%)	>1 SCA (%) ^a
Age		
18 months	90 (range 0–17.9, median 4.1 months) (85.7)	20 (22.2)
>18 months	15 (range 18.7–175.1, median 27.9 months) (14.3)	7 (46.7)
INSS		
1	61 (58.1)	12 (19.7)
2a	4 (3.8)	0 (0)
2b	24 (22.9)	7 (29.2)
2, not otherwise specified	16 (15.2)	8 (50)
Relapse		
No	89 (84.8)	17 (19.1)
Yes	16 (15.2)	10 (62.5)

^aPercent of the number with this characteristic.

TABLE III

Segmental Aberrations and Relapse Status in the Discovery Cohort

	No relapse	Local relapse	Metastatic relapse
Number of patients	6	11	9
1p deletion	0	1	0
2p gain	0	2	1
3p deletion	0	0	2
4p deletion	1	1	1
11q deletion	0	0	6
17q gain	0	1	6
NCA ^a	4	10	5
SCA ^a	1	4	6
Silent	2	1	1
Number of SCA (range)	1	1–3	5–11
Death from disease	0	0	2

^aFor the purposes of this table, NCAs and SCAs are counted as events; a tumor with both NCA and SCA is listed in both rows.