Table 1. Clinical Characteristics and 5-Year Overall Survival of 642 Patients With SMNs by Major Categories and Subtype

Type of SMN	Total		Males		ALL Immunophenotype* (n = 555)		Age at ALL (years)		WBC at ALL ($\times 10^9$/L)		Interval to SMN (years)		Age at SMN (years)		5-Year Survival Rate After SMN (%)
	No.	%	No.	%	BCP	%	Median	50% Range	Median	50% Range	Median	50% Range	Median	50% Range	
Total	642		346	53.9	434	78.2	5.2	3.2-10.3	11.4	4.7-45.0	4.8	2.6-8.9	12.6	7.8-17.5	40.4 ± 2.1†
Hematologic	345	53.7	198	57.4	234	79.6	5.2	3.2-11.2	9.0	4.2-37.0	2.9	2.0-4.5	9.4	6.5-15.2	35.2 ± 2.7
Acute myeloid leukemia	186		106	57.0	116	73.4	5.6	3.3-11.2	11.6	4.2-45.0	2.7	1.8-4.5	9.5	6.4-15.0	18.1 ± 2.9
Myelodysplastic syndrome	69		32	46.4	54	91.5	5.2	3.1-12.2	6.0	3.8-12.7	3.3	2.6-4.6	9.7	6.9-15.9	31.1 ± 6.2
Chronic myeloid leukemia	9		4	44.4	7	100.0	12.5	4.2-15.1	9	4.0-28.5	4.1	3.5-7.2	18.0	17.4-19.3	62.2 ± 17.8
Non-Hodgkin lymphomas	56		39	69.6	39	83.0	4.7	3.0-8.6	11.2	4.3-31.8	2.3	1.5-4.0	7.8	5.5-12.1	68.5 ± 6.4
Hodgkin disease	25		17	68.0	18	78.3	4.2	3.0-9.2	7.4	5.0-45.0	4.1	2.6-5.3	10.2	6.9-14.9	91.1 ± 6.0
CNS tumor	138	21.5	67	48.6	94	78.3	4.2	2.6-8.7	15.7	6.1-59.0	8.6	6.8-11.2	14.7	11.0-19.2	25.9 ± 4.2
Nonmeningioma CNS tumor	116		53	45.7	79	77.5	4.4	2.7-8.7	18.7	6.9-82.8	8.1	6.5-9.8	13.9	10.5-16.5	18.3 ± 3.8
Meningioma	22		14	63.6	15	83.3	3.5	2.3-8.5	9	5.1-30.0	16.2	12.3-18.3	21.7	17.8-25.4	90.9 ± 8.7
Carcinoma	78	12.1	34	43.6	62	84.9	5.8	3.3-10.6	12.3	4.0-45.6	10.1	6.7-14.5	17.5	12.4-22.2	82.2 ± 4.9
Nonthyroid carcinoma	46		19	41.3	35	81.4	8.4	3.9-13.0	12.9	3.6-38.5	10.2	6.1-15.0	18.0	12.4-25.8	67.3 ± 8.2
Thyroid carcinoma	32		15	46.9	27	90.0	5.0	3.1-6.5	12.1	4.3-58.5	10.1	7.8-13.5	15.5	12.1-18.3	100
Other	81	12.6	47	58.0	44	64.7	5.7	4.0-10.4	14.0	4.9-79.9	6.8	3.4-10.0	14.1	8.2-17.9	55.3 ± 6.1
Soft tissue sarcoma	29		14	48.3	14	60.9	6.0	4.1-10.4	19.8	7.3-68.0	5.4	3.3-9.6	13.3	8.0-17.2	43.9 ± 9.7
Bone tumor	22		13	59.1	14	77.8	5.3	2.9-8.1	7.0	3.1-30.9	7.8	5.2-11.4	14.4	11.9-17.9	61.9 ± 11.6
Melanoma	11		6	54.6	9	90.0	10.0	5.7-13.9	10.0	4.7-30.9	10.0	6.3-17.8	19.2	16.7-24.3	85.7 ± 13.2
Germ cell tumor	4		4	100.0	3	100.0	12.7	8.1-15.2	7.8	2.6-13.2	12.3	8.4-19.8	22.9	20.2-31.4	100
Histiocytosis	12		9	75.0	2	16.7	4.2	2.5-5.5	141.0	40.4-248.5	2.3	1.4-3.9	6.9	6.0-8.2	46.6 ± 14.8
Other	3		1	33.3	2	100.0	9.9	4.1-12.3	4.0	2.2-148.0	7.6	3.3-9.8	16.5	13.9-17.5	33.3 ± 27.2

Abbreviations: ALL, acute lymphoblastic leukemia; BCP, B-cell precursor; SMN, second malignant neoplasm.
*In all, 87 patients were excluded because immunophenotype was not reported (n = 75) or was not specified as either BCP or T-cell ALL (n = 12).
†Ten-year survival rate was 38.7% ± 2.2%.

Fig 1. Kaplan-Meier estimates of the interval between diagnosis of acute lymphoblastic leukemia (ALL) and development of the four major categories of second malignant neoplasms.

Thirty-eight (76.0%) of 50 patients with t-MN with an aberrant karyotype and previous exposure to epipodophyllotoxins had 11q23/*MLL* rearrangements, whereas only four (8.0%) had monosomy 7 and none had 5q−. In contrast, among the 46 patients with t-MN (52.2%) who had not been exposed to epipodophyllotoxins, 24 developed monosomy 7 (n = 20) or 5q− (n = 4) t-MN, and only 13 (28.3%) had 11q23/*MLL* rearrangements ($P < .001$).

Among patients who did not receive irradiation, 44 (79%) of 56 patients with solid tumors had previously received cyclophosphamide compared with 82 (57%) of 143 patients with hematologic malignancies or CNS tumors ($P = .005$).

Among the patients who did not receive transplantation for whom data on maintenance therapy methotrexate (n = 431) and mercaptopurine dosage (n = 422) were available, the patients who developed t-MN received higher starting doses of methotrexate and mercaptopurine than did patients who developed other SMNs ($P < .001$ for both drugs), and this was the case for both CNS patients who received irradiation ($P < .001$ and $P = .001$, respectively) and those who did not ($P = .007$ and $P = .02$, respectively). Thus, compared with patients with other SMNs, the patients who developed t-MNs were more likely to have received methotrexate starting doses of at least 25 mg/m^2 per week (45% *v* 28%; $P < .001$) and mercaptopurine starting doses of at least 75 mg/m^2 per day (52% *v* 29%; $P < .001$).

Neither the distribution of the four major categories of SMNs ($P = .37$) nor the time interval to SMN ($P = .84$) differed significantly between patients with low (n = 13; 10 by genotype and three by phenotype) versus normal (n = 114) thiopurine methyltransferase activity. Among the 413 patients who did not undergo transplantation but who did have data on the total duration of therapy, 65 (31.3%) of the 208 patients with t-MN and 36 (17.6%) of the 205 patients with solid tumors had received ALL therapy for 2.5 years or longer ($P = .001$).

Transplantation during first remission of ALL had been performed in 29 (5.7%) of the 510 ALL patients with available information. One (1.4%) of 74 patients with CNS tumors and seven (3.6%) of 193 patients with t-MN had received transplantation compared with nine (28.1%) of 32 patients with carcinomas and eight (15.4%) of 52 with other SMNs ($P < .001$).

Survival After SMNs

The median follow-up after diagnosis of an SMN was 4.9 years for the 292 patients who were alive at their latest follow-up. In all, 350 patients died within 20.6 years from diagnosis of an SMN (median, 0.75 years; 25th to 75th percentile: 0.4 to 1.4). The overall cumulative probability of death as a result of any cause was 59.6% ± 2.1% at 5 years and 61.3% ± 2.2% at 10 years after an SMN (Table 1 and Fig 2). The 10-year cumulative incidence of death as a result of the second (n = 236) or third (n = 1) cancer was 41.1% ± 2.1%; it was 5.6% ± 1.0% for relapsed ALL (n = 31), 10.4% ± 1.3% for treatment-related toxicities among patients who received a transplantation (n = 39) and those who did not (n = 20), and 4.2% ± 0.9% for unknown causes (n = 23; Fig 3). The 10-year probability of survival was 18.9% ± 6.9% (n = 33) for patients whose SMN occurred before 1990 (n = 54), 34.8% ± 2.8% (n = 296) for patients with SMNs diagnosed between 1990 and 1999, and 40.9% ± 6.3% (n = 313) for patients diagnosed from 2000 onward ($P < .001$).

Hematologic Malignancies

Survival remained consistently lower for patients with AML compared with those who had MDS ($P < .001$). The 5-year survival estimate for AML was 11.2% ± 2.9% for 125 patients diagnosed before 2000 and 34.1% ± 6.3% for 61 patients diagnosed after 2000 ($P < .001$). For MDS, the 5-year survival was 17.1% ± 6.4% for 36 patients diagnosed before 2000 and 48.2% ± 10.6% for 33 patients diagnosed after 2000 ($P = .005$). In a Cox regression model, adjusting for sex and age at diagnosis of SMNs and the use of CNS irradiation for ALL treatment, the improved outcome after 2000 was confirmed for both AML (estimated hazard ratio [HR], 0.62; 95% CI, 0.42 to 0.90; $P = .01$) and MDS (HR, 0.30; 95% CI, 0.15 to 0.60; $P < .001$). The hazard of death after t-MN decreased by approximately 10% for every additional year of interval between ALL and AML (HR, 0.88; 95% CI, 0.80 to 0.96; $P = .004$) with a similar trend for MDS (HR, 0.92; 95% CI, 0.80 to 1.06; $P = .23$).

For 185 patients with available information on transplantation after t-MN, the 5-year survival was 30.3% ± 4.4% for the 119 patients who received a transplantation and 11.4% ± 4.0% for the 66 who did not ($P < .001$). However, with a landmark at the median waiting time to transplantation of 4.1 months from SMN diagnosis, the 5-year survival estimates for patients who had received a transplantation and those who had not did not differ (26.7% ± 4.2% and 27.2% ± 7.7%, respectively),[28,31] and this was also the case for 78 patients with t-MN diagnosed in 2000 or later (42.0% ± 7.6% *v* 46.9% ± 11.5%). Among the patients with t-MN who received a transplantation, the 10-year survival for 30 patients with 11q23/*MLL* rearrangements (24.7% ± 8.3%) did not differ significantly from that of 26 patients with monosomy 7 (28.0% ± 9.0%).

Only two of the 25 patients with Hodgkin lymphoma died, both of whom were diagnosed with Hodgkin lymphoma in the 1980s. Excluding patients who received transplantation as part of their ALL therapy, the 5-year survival was 70.5% ± 7.9% for the 34 patients with non-Hodgkin lymphoma diagnosed in the 1990s and 65.4% ± 10.8% for the 22 patients diagnosed later ($P = .64$). The 5-year survival was 76.9% ± 8.3% for the 27 patients who had developed mature B-cell non-Hodgkin lymphoma.

Table 2. Pattern of SMNs in Relation to Their First-Line ALL Treatment in Patients Who Did Not Receive Hematopoietic Stem-Cell Transplantation

Type of Second Cancer	CNS Irradiation* (n = 432)		Epipodophyllotoxin* (n = 446)		Cyclophosphamide*				6-Mercaptopurine†			
					CNS Irradiation (n = 228)		No CNS Irradiation (n = 199)		CNS Irradiation (n = 230)		No CNS Irradiation (n = 192)	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Total	230	202	185	261	186	42	126	73	53	177	94	98
Hematologic SMN	79	145	105	127	67	11	82	61	25	50	76	61
t-MN was AML or MDS	64	109	84	96	54	9	60	47	22	38	61	43
CNS tumors	97	12	48	63	76	20	7	5	24	68	5	7
Non-CNS solid tumors	54	45	32	79	43	11	37	7	4	49	13	30

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; SMN, second malignant neoplasm; t-MN, therapy-related myeloid neoplasia.
*Only patients who did not receive transplantation who had available information on their therapy are included.
†Dose ≥ 75 mg/m².

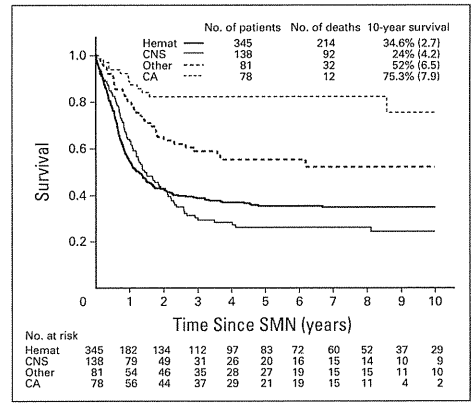

Fig 2. Survival curves according to the four major categories of second malignant neoplasms (SMNs). Hemat, hematologic; CA, carcinoma.

CNS Tumors

Although only one of 22 patients with meningioma died, the 5-year survival was very poor for the remaining 116 patients with brain tumors (18.3% ± 3.8%), including eight patients with low-grade tumors (45.0% ± 18.8%), 76 with high-grade tumors including medulloblastomas and supratentorial primitive neuroectodermal tumors (6.5% ± 3.6%), and 13 unspecified glial tumors (8.5% ± 8.2%). Overall survival after nonmeningioma brain tumor did not improve over time, with 5-year estimates of 19.6% ± 5.5% before 2000 and 16.6% ± 5.3% afterward ($P = .76$).

Nonthyroid Carcinomas

All seven patients with basal cell carcinoma and nine with parotid gland tumors survived, and the 5-year survival for the nine patients with squamous cell carcinoma was 71.4% ± 17.1%. In contrast, the overall survival for the 18 patients with other carcinomas (five, breast; four, gastrointestinal; three, liver; and one each, peritoneal, pancreas, lung, cervix uteri, urinary tract, and nasopharyngeal) was only 40.1% ± 13.7% at 5 years and 0% at 10 years ($P < .001$).

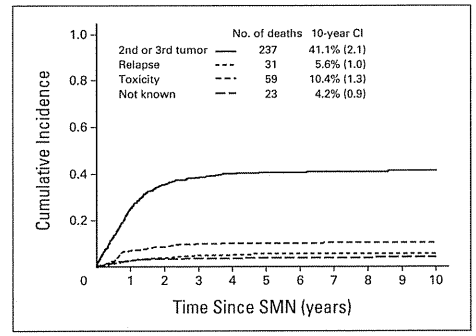

Fig 3. Cause-specific cumulative incidences (CIs) of death after development of a second malignant neoplasm (SMN).

DISCUSSION

In this study, the largest reported to date, patients with t-MN or nonmeningioma brain tumor had a poor prognosis, whereas patients with secondary meningioma, Hodgkin lymphoma, thyroid carcinoma, basal cell carcinoma, and parotid gland carcinoma had a 5-year survival exceeding 90%.

This study had some limitations since it did not allow calculations of HRs by ALL characteristics or therapy components, and it could not identify exposures that had equal influence on the risk of all major categories of SMNs. In addition, the data must be interpreted cautiously, since the completeness of recording of SMNs was influenced by the individual study groups' frequency and duration of follow-up,[1] screening strategies for thyroid carcinomas, meningiomas, or breast cancer in irradiated patients,[32-34] and linkage with population-based nationwide cancer registries.[18] The impact of such differences will be limited for secondary hematologic malignancies but will be more profound for SMNs that have long latency such as carcinomas and meningiomas. Furthermore, hematologic SMNs can be misinterpreted as relapse of ALL, and some cases of ALL and SMNs may have a common clonal origin.[35,36] Thus, an association between T-cell ALL and histiocytosis has previously been reported,[35,36] and patients with early T-cell precursor ALL have been shown to have genetic profiles similar to those of patients with myeloid malignancies,[37] which could indicate a common ancestral clone for the primary and second malignancies.

The observed association between high-hyperdiploid ALL and the development of t-MN with monosomy 7/5q– has been observed in a much smaller study,[2] although the association between ALL with specific chromosomal translocations (ie, t(9;22)(q34;q11.2), t(1;19)(q23;p13.3), t(12;21)(p13;q22)) and t-MN with 11q23/*MLL* rearrangements has hitherto not been reported. The more frequent use of topoisomerase II inhibitors such as epipodophyllotoxins in high-risk ALL cases with specific chromosomal translocation might have contributed to the development of t-MN with 11q23/*MLL* rearrangements. However, the unique gene expression profiles of ALL blast from those patients who subsequently developed SMNs, including t-MN, could also reflect inherited genetic variants[38] that could influence drug disposition (eg, glutathione S-transferases, cytochrome P-450 enzymes, quinone oxidoreductase, or the folate pathway[39,40]) or be related to cancer predisposition syndromes. International collaboration with extensive mapping of host genomic variants could be instrumental in identifying subsets of patients with ALL with genetic predispositions for whom modification of first-line ALL therapy or individualized follow-up should be offered.

This study supports previously reported associations of t-MN with higher mercaptopurine dosages during maintenance therapy and longer duration of therapy. Some study groups that offer a maintenance therapy mercaptopurine starting dose of 75 mg/m² have found an association between an increased risk of SMN and low-activity thiopurine methyltransferase genotypes or phenotypes.[2,41] Notably, others who used a mercaptopurine starting dose of only 50 mg/m² failed to find such an association.[42] The linkage between thiopurine

therapy and risk of SMN may reflect that these anticancer agents, when given at high dosage or for an extended period, may interfere with DNA repair rather than directly induce mutations.[41,43] Accordingly, the omission or interruption of maintenance therapy for patients who received a transplantation as part of their ALL therapy may explain why very few patients with brain tumor or t-MN in this cohort had received transplantation. Overall, the risk of relapse if mercaptopurine/methotrexate-based maintenance therapy is truncated[44] is far higher than the risk of t-MN indicated by this and previous studies. The goal for future research is thus to identify patients with a clearly excessive risk of t-MN and consider treatment modification only for such a limited patient subset.

Patients with t-MN have had significant improvements in survival over the last few decades, but the cure rates are still below those obtained by the best treatment protocols for primary AML.[45] Although the survival of patients with t-MN who did not receive transplantation was only 11.4% ± 4.0%, the study did not support that hematopoietic stem-cell transplantation would be beneficial for these patients when the data were adjusted for the waiting time to transplantation. Thus, future studies of this important issue, including the impact of t-MN cytogenetics, are needed.

It is uncertain whether the extremely poor survival rate for CNS tumors, the vast majority of which developed after CNS irradiation, reflects a more aggressive biology, difficulties in performing complete tumor resection in previously irradiated regions, limitations in irradiating previously irradiated regions, or a pessimistic attitude toward curative therapy for such patients. Because this subset is the second most common SMN among survivors of childhood ALL and is overall one of the most common SMNs after a childhood cancer,[18] a review of patients' records of these tumors is needed to explore these issues in depth.

Although the cure rates for some SMNs were as favorable as those obtained for their primary cancer counterparts, future strategies should continue to focus on prevention of SMNs. Thus, the frequency of secondary brain tumor is expected to fall dramatically during the coming decades with the reduced use of CNS irradiation in first-line ALL therapy,[46] and given the few patients on contemporary protocols who are exposed to epipodophyllotoxins, the risk of 11q23/*MLL*-rearranged t-MN is likely to be lower in future childhood ALL cohorts.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Appendix

Table A1. SMNs Reported by the Seventeen Participating Collaborative Groups

Trial Group Name	Trial Group Acronym	Trial Group Location	No. of Patients	Date of Diagnosis of First SMN	Date of Diagnosis of Last SMN	Trial Registration Numbers
Associazione Italiana Ematologia Oncologia Pediatrica	AIEOP	Italy	22	January 4, 1985	December 11, 2007	ALL-BFM 90, ALL-BFM 95, ALL-BFM 2000 (NCT00430118)
Berlin-Frankfurt-Münster	BFM	Austria	14	September 1, 1992	June 26, 2009	ALL-BFM 86, ALL-BFM 90, ALL-BFM 95, ALL-BFM 2000 (NCT00430118)
Berlin-Frankfurt-Münster	BFM	Germany	107	December 12, 1984	February 1, 2009	ALL-BFM 2000 (NCT00430118), NCI Protocol ID 68529
Cooperative Study Group for Childhood Acute Lymphoblastic Leukaemia	COALL	Germany	36	May 10, 1984	July 19, 2007	COALL 07-03, EU-205104, NCT00343369
Children's Oncology Group (includes both the US Children's Cancer Group and the Pediatric Oncology Group)	COG	USA	136	April 4, 1990	February 12, 2008	Separate list of POG and CCG protocols
Dutch Childhood Oncology Group	DCOG	Holland	18	February 26, 1991	May 30, 2008	
Dana-Farber Cancer Institute	DFCI	USA	13	August 14, 1986	March 17, 2008	DFCI ALL Consortium Protocols 85-001, 87-001, 91-001, 96-001
European Organisation for Research and Treatment of Cancer	EORTC	Belgium and France	16	June 30, 1991	June 15, 2002	EORTC 58881 study
French Acute Lymphoblastic Leukaemia Study Group	FRALLE	France	52	March 12, 1991	June 15, 2010	FRALLE protocols 83, 87-89, 93, 2000
Israel National ALL Studies	INS	Israel	11	June 16, 1993	December 15, 2008	ALL INS 89 (mod BFM 86), ALL INS 93 (mod BFM 90), ALL INS 98 (mod BFM 95)
Tokyo Children's Cancer Study Group	TCCSG	Japan	49	June 23, 1987	May 6, 2010	TCCSG L84-11, L89-12, L92-13, L95-14
Japan Association of Childhood Leukemia Study	JACLS	Japan				Tokai-POG 9104, OCLSG 94, JACLS ALL-96, JACLS ALL-97
Japanese Children's Cancer and Leukemia Study Group	JCCLSG	Japan				CCLSG ALL841, ALL851, ALL874, ALL911, ALL941
Kyushu-Yamaguchi Children's Cancer Study Group	KYCCSG	Japan				KYCCSG AL841, HR88, ALL90, ALL96
Nordic Society for Paediatric Haematology and Oncology	NOPHO	Denmark, Finland, Iceland, Norway, Sweden	53	January 15, 1986	May 15, 2010	ALL-86, ALL-92, ALL-2000
St Jude Children's Research Hospital	SJCRH	USA	69	February 9, 1982	November 18, 2002	Total Therapies 4, 5, 6, 7, 8, 9, 10, 11, 12, 13A, and 13B
Taiwan Pediatric Oncology Group	TPOG	Taiwan	19	August 5, 1987	January 13, 2007	TCALL 84; TPOG-ALL 88, 93, 97, 2002
National Cancer Research Institute Children's Leukaemia Clinical Studies Group	NCRI	United Kingdom	27	January 15, 1994	September 15, 2007	UKALLXI ISRCTN 16757172, ALL97 ISRCTN 26727615
Total			642	February 9, 1982	June 15, 2010	

Table A2. Clinical Characteristics of Patients With Cancer-Predisposing Syndromes

Predisposing Syndrome	Type of Second Cancer	Sex	Age at ALL (years)	WBC at ALL ($\times 10^9$/L)	BCP or T-Cell ALL	Interval to SMN (years)	Age at SMN (years)	Status	Survival (years)
Down syndrome	AML	Male	3.2	16.8	B	4.0	7.2	Dead	0.8
Down syndrome	AML	Female	2.0	7.8	B	5.9	7.9	Dead	1.1
Down syndrome	Mature B-cell NHL	Male	6.2	38.1	B	2.6	8.8	Alive	7.0
Down syndrome	Ewing sarcoma	Female	6.6	2.1	B	8.3	14.9	Alive	5.4
Li Fraumeni syndrome	AML	Male	12.4	6.6	B	2.5	15.0	Dead	0.6
Ataxia telangiectasia	T-cell NHL	Male	9.5	86.0	T	12.5	22.0	Dead	0.6
Noonan syndrome	MDS	Female	16.0	2.0	B	2.7	18.7	N/A	
AIDS	Mature B-cell NHL	Male	13.7	1.8	B	4.0	17.7	Alive	10.2

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BCP, B-cell precursor; MDS, myelodysplastic syndrome; N/A, not available; NHL, non-Hodgkin lymphoma; SMN, second malignant neoplasm.

Table A3. Clinical Characteristics and Overall Survival of the Four Major Categories of SMNs in the Subset of 201 Patients Who Were Not Irradiated and Did Not Undergo Hematopoietic Stem-Cell Transplantation as Part of Their First-Line Treatment for ALL

Type of Second Cancer	Total		Males		ALL Immunophenotype* (n = 192)		Age at ALL (years)		WBC at ALL ($\times 10^9$/L)		Interval to SMN (years)		Age at SMN (years)		5-Year Survival Rate After SMN (%)
	No.	%	No.	%	BCP	%	Median	50% Range	Median	50% Range	Median	50% Range	Median	50% Range	
Total	201		107	53.2	173	90.1					3.6	2.3-6.6	9.0	6.5-15.1	44.1 ± 3.7
Hematologic†	145	72.1	79	54.5	130	92.2	4.3	3.0-6.5	6.1	4.0-15.3	2.9	2.1-4.3	8.2	6.0-12.7	41.1 ± 4.2
CNS tumor†	12	6.0	6	50.0	9	90.0	5.0	3.5-8.9	7.4	3.7-34.4	6.8	2.7-7.4	13.1	8.7-17.2	32.1 ± 15.0
Carcinoma†	19	9.5	7	36.8	15	83.3	4.7	3.0-8.7	6.6	3.3-38.5	11.8	6.1-16.1	16.2	10.7-23.4	77.4 ± 10.0
Other†	25	12.4	15	60.0	19	82.6	5.7	3.4-8.1	4.9	2.5-26.2	7.8	4.4-9.8	14.0	10.4-17.9	44.9 ± 11.3

Abbreviations: ALL, acute lymphoblastic leukemia; BCP, B-cell precursor; SMN, second malignant neoplasm.
*Nine patients were excluded because immunophenotype was not reported (n = 8) or was not specified as either BCP or T-cell ALL (n = 1).
†Seventy-one acute myeloid leukemia, 38 myelodysplastic syndrome, three chronic myeloid leukemia, 23 non-Hodgkin lymphoma, 10 Hodgkin disease, 10 nonmeningioma CNS tumors, two meningioma, 10 nonthyroid carcinoma, nine thyroid carcinoma, seven soft tissue sarcoma, 12 bone tumors, one germ cell tumor, four Langerhans cell histiocytosis, one other tumor.

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PRIORITY REPORTS
Clinical Characteristics and Outcomes of Chédiak–Higashi Syndrome: A Nationwide Survey of Japan

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Background. Chédiak–Higashi syndrome (CHS) is a rare autosomal recessive disorder characterized by immunodeficiency, neurological dysfunction, and oculocutaneous albinism. Recently, several clinical CHS phenotypes have been reported. Here, we report results of a nationwide survey performed to clarify clinical characteristics and outcomes of CHS patients in Japan. ***Methods.*** Questionnaires were sent to 287 institutions to collect data regarding CHS patients diagnosed between 2000 and 2010, including results of lysosomal trafficking regulator (*LYST*) gene analysis. Cytotoxicity and degranulation activity of cytotoxic T lymphocytes were analyzed in available patient samples. ***Results.*** A total of 15 patients diagnosed with CHS were eligible for enrollment in this study. Of these, 10 (67%) had recurrent bacterial infections, five (33%) developed life-threatening hemophagocytic lymphohistiocytosis (HLH), and one patient had complicated malignant lymphoma. Hematopoietic stem cell transplantation (HSCT) was performed for six patients including three with HLH, and 10 of the enrolled patients have survived at the time of this writing. *LYST* analysis was performed for 10 patients; seven different mutations were detected in seven patients, whereas no mutation was identified in three patients. Cytotoxicity and degranulation activity were impaired in patients with and without *LYST* mutation. ***Discussion.*** Results of this survey indicate that one or two patients with CHS were newly diagnosed each year in Japan. The incidence of HLH was not as high as expected. Mutations of genes other than *LYST* were suspected in some cases. We conclude that determining indication for HSCT for CHS patients should be based on genetic and cytotoxic analysis. *Pediatr Blood Cancer* 2013;60:1582–1586.

Key words: Chédiak–Higashi syndrome; cytotoxic T lymphocyte; degranulation activity; lysosomal trafficking regulator (LYST) gene

INTRODUCTION

Chédiak–Higashi syndrome (CHS) is a rare autosomal recessive disorder characterized by immunodeficiency, neurological dysfunction (muscle weakness, ataxia, sensory loss, and nystagmus), oculocutaneous albinism, and a bleeding tendency due to impaired platelet function [1,2]. The hallmark of CHS is the occurrence of giant inclusion bodies in virtually all granulated cells, including granulocytes, histiocytes, mast cells, platelets, melanocytes, Schwann cells, neurons, renal tubular epithelial cells, and fibroblasts. Recent evidence suggests that lysosomes and melanosomes may originate via a shared intracellular pathway [3], and it thus seems likely that CHS is the result of a defective protein required for the normal genesis, structure, or function of a variety of intracellular organelles, including melanosomes, lysosomes, and intracellular secretory granules.

CHS is thought to be caused by mutation of the lysosomal trafficking regulator (*LYST*) gene localized in chromosome 1q42–44, which consists of 51 coding exons and encodes for a 429-kD protein [4]. The *LYST* gene produces a cytoplasmic protein that modulates lysosomal exocytosis. LYST interacts with some cytoplasmic proteins that play important roles in regulation of vesicular transport or signal transduction, such as hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), casein kinase II (CK2), calmodulin (CALM), and 14-3-3 proteins [5]. HRS inhibits exocytosis by binding SNAP25, a component of the SNARE protein complex that is important in vesicle docking and fusion [6–8]. LYST forms a complex with HRS, and the HRS–LYST complex is unable to bind SNAP25. CALM has a significant role in membrane fusion. Furthermore, mediation of HRS activity by complexes of LYST and CALM is thought to enhance SNAP25-regulated membrane docking and fusion. In CHS, the absence of LYST might prevent juxtaposition of HRS and CALM, potentiating HRS inhibition of SNAP25, thus inhibiting membrane docking and fusion [5]. These gene defects cause neutrophil digestion and migration, and are also cytotoxic when occurring in natural killer cells (NK-cells) and cytotoxic T lymphocytes (CTLs).

Recently, different clinical CHS phenotypes have been reported. Most patients develop HLH in an "accelerated phase" during infancy or childhood, whereas others have a milder course and survive into adulthood without developing HLH [9]. A recent study also demonstrated that cytotoxic function was significantly reduced in CHS patients who developed early-onset HLH [10]. To clarify the association between phenotypes and genotypes, we performed a nationwide survey of CHS in Japan. Functional analysis of CTLs was performed for two patients whose samples were available.

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METHODS

Patients

Questionnaires were initially sent to 287 institutions in Japan that employ a pediatric or adult hematologist, enquiring about CHS patients treated between 2000 and 2010. Of these institutions, 206 (71.8%) responded to the first questionnaire; a total of 18 patients had been diagnosed in 14 institutions. A second questionnaire was then sent to these 14 institutions, asking for information regarding clinical features, treatments, and outcomes for each patient. Finally, 15 patients were diagnosed with CHS by clinical manifestations including giant granules in blood cells, oculocutaneous albinism, recurrent infections, bleeding disorders and variable neurological involvement, and all were therefore eligible for this study. The institutional review board of Ehime University Graduate School of Medicine approved this study, and written informed consent was obtained from the parents or guardians of each patient.

Genetic Analysis of the LYST Gene

Genomic DNA was extracted from peripheral blood samples obtained from CHS patients. Polymerase chain reaction (PCR) primer pairs designed from intron sequences flanking each exon were used to amplify genomic DNA segments spanning each exon. PCR was performed according to the manufacturer's protocol (TOYOBO, Tokyo, Japan). Direct sequencing using dye termination cycle sequencing was performed at FASMAC Co., Ltd (Atsugi City, Kanagawa, Japan). The mutations were analyzed using Sequence Scanner Ver. 1 (Applied Biosystems, Tokyo, Japan) and were also checked at the cDNA level.

Analysis of CTL-Mediated Cytotoxicity

Alloantigen-specific $CD8^+$ CTL lines were generated as described previously [11]. Briefly, peripheral blood mononuclear cells (PBMCs) from an individual CHS patient and from healthy individuals were co-cultured with a mitomycin C (MMC)-treated B-lymphoblastoid cell line (B-LCL) established from an HLA-mismatched individual (KI-LCL). Then the $CD8^+$ T lymphocytes were isolated. $CD8^+$ T lymphocytes cultured with 10 IU/ml interleukin-2 were stimulated with MMC-treated KI-LCL cells three times at 1-week intervals, and were then used as $CD8^+$ alloantigen-specific CTL lines. The cytotoxic activity of the CTLs was measured by a standard ^{51}Cr-release assay, as described previously [12]. Briefly, alloantigen-specific CTLs were incubated with ^{51}Cr-labeled allogeneic KI-LCL cells or TA-LCL cells for 5 hours at an effector:target (E:T) cell ratio of 1.25:1, 2.5:1, and 5:1. Target cells were also added to wells to determine the spontaneous and maximal levels of ^{51}Cr release. The percentage of specific ^{51}Cr release was calculated as (cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release) × 100, where cpm indicates counts per minute.

Degranulation Analysis

Degranulation activity was analyzed by flow cytometry using anti-CD107a antibody (BioLegend, San Diego, CA) as described previously [13]. Briefly, 1×10^5 alloantigen-specific CTLs were co-cultured with or without 1×10^5 KI-LCL cells, and then FITC-conjugated anti-CD107a antibody was added to each culture well. After 3 hours, incubated cells were collected and analyzed by flow cytometry using PE-conjugated anti-CD8 antibody (BD Biosciences, Franklin Lakes, NJ). For analysis of degranulation, the relative log fluorescence of live cells was measured using a FACS flow cytometer (BD Biosciences). The immunofluorescence intensities of CTLs cultured with and without alloantigen stimulation were measured, and the mean fluorescence index (MFI) was calculated as (mean value for stimulated sample − mean value for non-stimulated sample)/mean value for non-stimulated sample.

RESULTS

Clinical Characteristics of CHS

The clinical characteristics of 15 patients with CHS are summarized in Table I. The mean age of diagnosis was 1.0 year (range, 0 months–23 years); seven were male and eight were female; and two patients (13%) had consanguineous parents. All 15 patients had giant granules in neutrophils, and all but one had oculocutaneous albinism. A total of 10 patients (67%) had recurrent bacterial infections, including pneumonia, enteritis, and otitis media, and 5 (33%) developed life-threatening "accelerated phase" HLH. Complicated malignant lymphoma was present in one patient (UPN9) (6%). Hematopoietic stem cell transplantation (HSCT) was performed for six patients including three with HLH; three with unrelated, and three with related donor bone marrow. At the time of this writing, in April 2013, 10 patients have survived and four have died (two of infection, one of liver failure, and one of amyloidosis). Of the six patients who underwent HSCT, all but one have survived.

Analysis of LYST Gene

Genetic analysis of *LYST* was performed in 10 patients. *LYST* gene mutations were detected in 7 of those 10 patients, and 5 of them have been reported previously by Tanabe et al. [14]. A 5541–5542delAA in one allele of exon 18 was found in three patients who were siblings. The other four patients were found to have the following mutations: 7982C > G/8281A > T; 10445–10446insCA/7207C > T; 3944insC/−; and 1044–1045insA/−. No *LYST* gene mutation was found in the other three patients. The patients without *LYST* mutation also displayed giant granules in neutrophils, as shown in Figure 1.

Cytotoxic and Degranulation Analysis

Alloantigen-specific $CD8^+$ CTL lines were generated from three healthy individuals and two CHS patients (UPN1 and UPN2). Antigen-specific cytotoxicity mediated by CTLs from the patient with a *LYST* nonsense mutation was significantly reduced compared with that of the healthy individuals, whereas that of the patient without a *LYST* mutation was only mildly impaired (Fig. 2). Degranulation activity mediated by CTLs was also measured (Fig. 3). Control CTLs showed a significant increase of fluorescence intensity following alloantigen stimulation, whereas the increase of fluorescence intensity in the two CHS patients was relatively low; the MFI of the three healthy individuals (mean [SD]) was 3.10 (0.75), and the MFIs of the two CHS patients analyzed were 1.22 and 1.37.

TABLE I. Clinical and Genetic Findings of 15 Patients With Chédiak–Higashi Syndrome

UPN	Age/gender	Age at diagnosis	Family history	Recurring infection	HLH development	Neurological dysfunction, other complications	NK-cell activity[a]	*LYST* gene mutation	HSCT	Outcome
1	22-year-old/female	1 year	No	None	Yes	Hypopituitarism, diabetes insipidus leukoencephalopathy	NT	7982C > G (nonsense), 8281A > T (nonsense)	—	Alive
2	23-year-old/male	10 years	No	Otitis media subcutaneous abscess	No	Parkinson's disease	NT	No mutation detected	—	Alive
3	23-year-old/male	6 months	Yes	None	No	None	2.9%	5541–5542delAA (frameshift)/—	—	Alive
4	20-year-old/female	1 year	Yes	None	No	None	7.3%	5541–5542delAA (frameshift)/—	—	Alive
5	17-year-old/female	1 month	Yes	None	No	None	7.4%	5541–5542delAA (frameshift)/—	—	Alive
6	25-year-old/female	18 years	No	Enteritis	No	Mental retardation amyloidosis	NT	NT	—	Dead (amyloidosis)
7	16-year-old/female	1 year	No	Yes	No	None	NT	NT	—	Alive
8	5-year-old/male	1 year	No	Yes	Yes	Cerebral atrophy	2.6%	10445–10446insCA (frameshift)/ 7207C > T (nonsense)	ABMT sibling donor	Alive
9	14-year-old/female	0 month	Yes	Subcutaneous abscess	Yes LAHS	Malignant lymphoma	NT	NT	UBMT	Alive
10	32-year-old/female	5 years	No	Respiratory infection, gingivitis	No	None	5%	No mutation detected	—	Dead (infection)
11	6-year-old/female	4 years	No	None	No	None	0.7%	3944insC (frameshift)/—	ABMT from mother	Dead (infection)
12	7-year-old/male	6 years	No CM	Otitis media	No	None	1%	NT	ABMT sibling donor	Alive
13	10-year-old/male	4 months	No	Yes	Yes	None	3%	NT	UBMT	Alive
14	9-year-old/male	2 years	No CM	Respiratory infection, otitis media	No	Peripheral nerve involvement	17%	No mutation detected	—	Dead (liver failure)
15	11-month-old/male	11 months	No	Enteritis	Yes	None	1.4%	1044–1045insA (splice mutation)/—	UBMT	Alive

UPN, unique patient number; CM, consanguineous marriage; LAHS, lymphoma associated hemophagocytic syndrome; NT, not tested; WT, wild type; HSCT, hematopoietic stem cell transplantation; ABMT, allogeneic bone marrow transplantation; UCBT, unrelated cord blood transplantation. [a]Normal value, >17% when effector:target ratio is 20:1.

Fig. 1. Giant granules in a neutrophil from CHS patient without *LYST* mutation. The patient without *LYST* mutation (UPN1) displayed giant granules in neutrophils.

DISCUSSION

In this nationwide survey of Japan, data from a total of 15 patients with CHS were collected and analyzed. Since this survey identified more than 80% of all possible cases during a 10-year period, we conclude that one or two patients with CHS will be newly diagnosed per year in Japan. The diagnosis of CHS was appropriate for all patients enrolled in this study, because all had giant granules in neutrophils and oculocutaneous albinism. Interestingly, the incidence of HLH among the CHS patients in this study was only 33%, which is much lower than that of previous reports [15]. The only treatment reportedly leading to cure of CHS has been HSCT [16]. Without HSCT, CHS is usually fatal before the age of 10 years because of "accelerated phase" HLH induced by infection [17]. It has been reported that patients without accelerated phase HLH can survive for more than 20 years. Of the patients enrolled in this study, six of nine who did not receive HSCT have survived. However, a major concern is that neurological dysfunction cannot be prevented by HSCT [18].

Fig. 2. Cytotoxicity of alloantigen-specific $CD8^+$ T-cell lines. $CD8^+$ T-cell lines were generated from two CHS patients and three healthy individuals by stimulation with allogeneic B-LCL (KI-LCL) cells. Cytotoxicity against allogeneic KI-LCL (clear circles) and against allogeneic TA-LCL (solid circles) that shared no HLA antigens with KI-LCL was determined. CTL-mediated cytotoxicity was impaired in the two patients, but was more severely impaired in UPN1, who developed HLH. HLA types of B-LCLs were as follows: KI-LCL, HLA-A01/30, B13/17, Cw6/–, DRB1*0701/*0701; TA-LCL, HLA-A24/26, B62/–, Cw4/w9, DRB1*0405/*0901.

Fig. 3. CD107a expression of alloantigen-specific $CD8^+$ T-cell lines. Flow cytometric analysis of CD107a expression in alloantigen-specific $CD8^+$ T-cell lines generated from a healthy individual and two CHS patients. The left panel of each column shows CD107a expression in unstimulated $CD8^+$ T cells. The right panel of each column shows CD107a expression in $CD8^+$ T cells stimulated with KI-LCL cells. Degranulation activity was also impaired in the two patients, but was more severely impaired in UPN1, who developed HLH. MFI indicates mean fluorescence index calculated as (mean value for stimulated sample – mean value for non-stimulated sample)/mean value for non-stimulated sample.

In this study, *LYST* analysis was performed in 10 patients, and seven different mutations were detected in seven patients. All these mutations were frame shift or nonsense mutations, resulting in loss of LYST function. Three of seven patients with *LYST* mutations had HLH at diagnosis, and received HSCT. Three other patients clinically diagnosed with CHS had no *LYST* mutation. Karim et al. [17] reported mutational analysis of *LYST* in 21 unrelated patients with CHS; no mutation was found in 10 patients, and mutation of one allele was seen in four. It is possible that other causative genes exist, or that the relevant mutation is located in the intron or splice mutation site in patients without *LYST* mutations *per se*. Recently, it was shown that synaptotagmin III (Syt III), a member of Syt family of proteins, deficient Rat basophilic leukemia cells displayed enlarged secretory granule, reminiscent of that in Chediak–Higashi (beige) mice [19]. CHS without *LYST* mutations might be caused by dysfunction of genes playing a critical role of the formation and delivery of internalized cargo to the perinuclear endocytic recycling compartment.

CHS includes several different phenotypes. Most of the patients develop accelerated phase HLH as during infancy or childhood,

while 10–15% of patients have a milder phenotype and survive to adulthood, but often develop fatal neurological dysfunctions [9]. It was reported that the clinical phenotype of CHS is correlated with molecular genotype; loss-of-function mutations are associated with severe, early-onset CHS, whereas missense mutations are associated with milder, late-onset CHS without HLH [9]. Recent studies also demonstrated that cytotoxicity of CTL was significantly reduced in patients with early-onset HLH, whereas those with later onset or no HLH had low normal CTL cytotoxicity [10]. In this study, impaired CTL cytotoxicity was analyzed in two patients, and the findings were different; the patient who developed HLH had severely impaired cytotoxic and degranulation activity, compared with the other patient who had no HLH.

The only therapy for CHS leading to cure is HSCT, which should be performed before the development of HLH. However, the precise indication for, and optimal timing of, HSCT remains unclear for individual patients, because of the existence of different subtypes and clinical courses. The current findings indicate that the cytotoxic function of CTLs and degranulation activity can predict the development of HLH and might be appropriate for use as an indication for HSCT in patients with CHS.

In conclusion, based on genetic and functional analysis, CHS is a rare but heterogeneous disorder affecting children and young adults. HSCT is the only treatment that can cure CHS, but it cannot prevent development of neurological dysfunction. We have recently established iPS (induced pluripotent stem cell) lines from patients with CHS to help clarify the pathogenesis of neurological dysfunction. It is our hope that in the near future, a novel strategy may be developed to prevent both HLH and the neurological dysfunction of CHS.

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Cancer-specific health-related quality of life in children with brain tumors

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Abstract
Purpose To understand the influence of disease and treatment on the health-related quality of life (HRQOL) of children with brain tumors, compared to the HRQOL of children with other cancers, from the viewpoints of children and parents.
Methods A total of 133 children aged 5–18 years and 165 parents of children aged 2–18 completed questionnaires of the Pediatric Quality of Life Inventory Cancer Module (Pain and Hurt, Nausea, Procedural Anxiety, Treatment Anxiety, Worry, Cognitive Problems, Perceived Physical Appearance, and Communication scales); higher scores indicate a better HRQOL. The Cancer Module scores, weighted by age and treatment status, were compared to those obtained in a previous study of children with other cancers (mostly leukemia).
Results The weighted mean scores for Pain and Hurt (effect size $d = 0.26$) and Nausea ($d = 0.23$) from child reports and the scores for Nausea ($d = 0.28$) from parent reports were higher for children with brain tumors than scores for children with other cancers. The scores for Procedural Anxiety ($d = -0.22$) and Treatment Anxiety ($d = -0.32$) from parent reports were lower for parents of children with brain tumors than the scores for parents of children with other cancers. The child-reported Pain and Hurt score of the Cancer Module was higher ($d = 0.29$) and in less agreement (*intraclass correlation coefficient* $= 0.43$) with scores from the Brain Tumor Module, indicating that assessments completed with the Cancer Module misesteem pain and hurt problems in children with brain tumors.
Conclusions The profiles of cancer-specific HRQOL in children with brain tumors differ from those of children with other cancers; we therefore suggest that these children receive specific psychological support.

Keywords Brain neoplasms · Child · Japan · Quality of life · Questionnaires

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Introduction

While modern treatment methodologies have improved the outcome for pediatric cancer survival to approximately 70–80 % [1, 2], managing health-related quality of life (HRQOL) during and after treatment becomes a more important part of treatment. Brain tumors are the second most common (27 %) form of pediatric cancer after leukemia (33 %) [3]. Children with brain tumors often experience pain, nausea, lack of energy, and emotional distress [4, 5] and may also experience late effects, such as endocrinological problems, cognitive impairment, neurological (motor and sensory) disability, and posttraumatic stress symptoms [6–8]. Consequently, survivors of brain tumors who receive intensive treatment [9, 10] are at higher risk of physical, psychological, social, and developmental difficulties than survivors of other cancers [11–14]. By understanding the HRQOL profile of these children, medical practitioners can design targeted interventions to maintain and improve HRQOL in this population during and after treatment.

Global profiles of HRQOL (for example, physical, emotional, and social) in children with brain tumors are lower than those of children with other cancers or without cancer [15–18]. However, little information is available on disease-specific HRQOL profiles in children with brain tumors. Meeske et al. compared cancer-specific HRQOL between children with brain tumors and those with acute lymphoblastic leukemia (ALL) using the parent-reported Pediatric Quality of Life Inventory (PedsQL) Cancer Module [17], finding that parents of children with brain tumors and acute lymphoblastic leukemia report different experiences for their children during and after treatment. This highlights the need to understand how children with brain tumors perceive their own HRQOL.

The disease-specific HRQOL of patients with brain tumors can be measured with one of several cancer-specific tools [19–21], such as the PedsQL Cancer Module, or with a brain-tumor-specific tool [15, 22, 23], such as the PedsQL Brain Tumor Module. Different tools may provide different measures of HRQOL, as the questionnaire structure, number, and time of the questions differ among available tools. Here, we compared cancer-specific HRQOL in children with brain tumors with the HRQOL of children with other cancers, the reported views of children and their parents, and the HRQOL as measured by two PedsQL modules—the PedsQL Cancer and the PedsQL Brain Tumor Modules.

Methods

This study was conducted jointly with the development of the Japanese version of the PedsQL Brain Tumor Module [24].

Study population

Children with brain tumors and their parents were recruited from six hospitals across Japan and from the Children's Cancer Association of Japan (CCAJ) between September and December 2008. Inclusion criteria were as follows: age 5–18 years for children (the parent was included if their child was 2–18 years) and at least 1 month had passed since diagnosis. Children and parents were excluded if physicians at the hospital or social workers of the CCAJ determined that the family found the subject of the child's condition too uncomfortable to discuss.

Procedure

Researchers presented the study aims to 101 children and 122 parents at participating hospitals verbally and in writing, and the CCAJ sent a written notice to all families, inviting them to a meeting regarding brain tumors. Of 55 families from the CCAJ that provided informed consent or assent, 2 families were bereaved, 1 had an adult survivor, 6 children were aged 2–4 years, and 1 child old enough to provide his own consent opted out. A total of 98 children and 120 parents from the hospitals as well as 45 children and 52 parents contacted directly by the CCAJ agreed to participate. Questionnaires were distributed to 143 children and 172 parents.

Questionnaires for children were either self-administered or administered by an interviewer. When providing

informed consent, parents determined whether or not their child was able to self-administer the questionnaire. In accordance with the PedsQL™ administration guidelines, children aged 5–7 years or who were otherwise determined incapable of self-administration were administered the questionnaire by either their parents or a researcher (children were allowed to decide). In both cases, the instructions and each item were read to the child. Parent report questionnaires were simultaneously self-administered.

The questionnaires were returned by 138 children and 167 parents. We excluded questionnaires from 5 children and 2 parents who did not answer any scales of the PedsQL Cancer Module, and we analyzed answers from 133 children and 165 parents. Next, we analyzed answers from 124 children and 143 parents after omitting questionnaires with missing data for any scale of the PedsQL Cancer Module. Given the lack of any significant differences between the results of the former and latter analyses, we report only the latter.

Ethical considerations

This study was approved by the review boards of all seven participating institutions. Children aged ≥12 years and the parents of all children provided written consent prior to participation. Children aged <12 years provided informed verbal assent.

Measurements

The cancer-specific HRQOL of the PedsQL Cancer Module [21, 25] has eight scales: Pain and Hurt (two items), Nausea (five items), Procedural Anxiety (three items), Treatment Anxiety (three items), Worry (three items), Cognitive Problems (five items), Perceived Physical Appearance (three items), and Communication (three items).

Respondents were asked to describe the extent to which each item troubled them over the past month. Although the PedsQL Cancer Module comprises the standard (covering the previous month) and acute versions (covering the previous 7 days), we used the standard version, because it served as a historical control (described in the next section). For the child reports for ages 8–18 and all parent reports, a 5-point Likert response scale was used (0 = never a problem; 1 = almost never; 2 = sometimes; 3 = often; 4 = almost always). For the child report for children ages 5–7, a 3-point face scale was used. Items were reverse scored and linearly transformed to a 0–100 scale, with higher scores indicating a better HRQOL. To account for missing data, scale scores were computed as the sum of the items divided by the number of items answered. If more than 50 % of the items were missing or incomplete, the scale score was not computed.

Table 1 Characteristics of participants

	This study				Tsuji et al. [25] ($N = 245$)	
	All participants ($N = 165$)		Complete participants ($N = 143$)[a]			
	n	%	*n*	%	*n*	%
Gender						
Male	91	55.5	84	59.2	135	55.1
Female	73	44.5	58	40.8	110	44.9
Age (years)						
2–4	25	15.2	23	16.1	41	16.7
5–7	31	18.8	21	14.7	62	25.3
8–12	56	33.9	48	33.6	75	30.6
13–18	53	32.1	51	35.7	67	27.3
Tumor pathology						
Embryonal tumors	47	29.2	39	27.9	–	–
Germ cell tumors	36	22.4	34	24.3	–	–
High-grade glioma	24	14.9	19	13.6	–	–
Low-grade glioma	39	24.2	33	23.6	–	–
Other tumors	15	9.3	15	10.7	–	–
Treatment status						
On-treatment	63	39.4	56	39.2	88	35.9
Off-treatment ≤ 12 months	23	14.4	21	14.7	33	13.5
Off-treatment > 12 months	74	46.3	66	46.2	124	50.6
Age of guardian (years)						
21–28	7	4.3	4	2.8	5	2.1
29–34	23	14.0	18	12.7	40	16.9
35–39	47	28.7	41	28.9	72	30.4
40–60	86	52.4	78	54.9	120	50.6
≥61	1	0.6	1	0.7	0	0.0
Relationship to patient						
Mother	152	92.1	133	93.0	230	96.2
Father	10	6.1	8	5.6	9	3.8
Other guardian	3	1.8	2	1.4	0	0.0
Guardian's academic background						
Junior high school	3	1.9	2	1.4	4	1.7
High school	63	38.9	49	35.0	87	36.6
Vocational school	28	17.3	27	19.3	44	18.5
Junior college	29	17.9	28	20.0	48	20.2
University	36	22.2	32	22.9	52	21.8
Graduate school	3	1.9	2	1.4	1	0.4
Other	0	0.0	0	0.0	2	0.8

Missing data were excluded

[a] Sample without missing data for any scale of the PedsQL Cancer Module

The PedsQL Brain Tumor Module [15, 24] has six scales. Questions about Nausea, Procedural Anxiety, and Worry scales are identical to those in the PedsQL Cancer Module, whereas questions on the Pain and Hurt scale (three items) and Cognitive Problems scale (seven items) differ from those in the PedsQL Cancer Module. The parent report for toddlers (ages 2–4) does not include the Cognitive Problems scale. The Movement and Balance scale is not reported here. Agreement between the parent and child reports (intraclass correlation coefficient [ICC]) was described previously as follows: 0.41 (Pain and Hurt), 0.65 (Nausea), 0.62 (Procedural Anxiety), 0.18 (Worry), and 0.49 (Cognitive Problems) [24].

Respondents were asked to describe the extent to which each item troubled them over the previous 7 days. Although the recall period of the questionnaire differed from that of the Cancer Module, no published studies using the Brain Tumor Module as the standard (1 month) version were available when the present study was planned and designed. Because the PedsQL Brain Tumor Module adopts the acute version (covering the previous 7 days) as a standard, we employed the acute version. The respondents, response scale, and scoring method were identical to the PedsQL Cancer Module. Parents were also asked to record their child's gender, date of birth, age, tumor pathology, date of diagnosis, and date of therapy completion.

Historical control

We used data reported by Tsuji et al. [25] as a control. This study reported scores from for Japanese children with cancer (67.8 % had leukemia, 9.0 % had malignant lymphoma, followed by neuroblastoma, Wilm's tumor, rhabdomyosarcoma, and hepatoblastoma) using the Japanese version of the PedsQL Cancer Module. Children with brain tumors were excluded in that study.

The average age of children with cancer was 10.5 years (standard deviation [SD] = 3.9 years), and 55.1 % of patients were boys (Table 1). Mothers answered 93.9 % of the questionnaires, and parents' ages ranged between 40 and 60 years.

Statistical analysis

Statistics were calculated using IBM SPSS software, version 19 (SPSS, Inc., Chicago, IL, USA), and the level of significance was defined as 0.05. We calculated the sample characteristics as follows: age distribution, disease, and treatment characteristics; and scale characteristics as follows: mean, SD, minimum and maximum scores. The internal consistency of each subscale was estimated using Cronbach's alpha coefficient [26] (good consistency > 0.70). The agreement between the child and parent reports was estimated using ICC in a two-way mixed effects model [27] (ICC value of 0.20 indicates fair agreement, 0.40 moderate, 0.60 good, and 0.80 high agreement).

The cancer-specific HRQOL of children with brain tumors was compared to the HRQOL of children with other cancers. We compensated for the effect of age (toddler, young child, school child, or adolescent) and treatment status (on-treatment, soon after treatment, or off-treatment) differences using the weighted means and SDs of the PedsQL Cancer Module scale scores, adjusted for age and treatment status. The age distribution of leukemia and brain-tumor onset differs [29, 30], and previous reports have found that treatment status affects the PedsQL Cancer Module score [21, 25]. We also found in this study that the treatment status affected the PedsQL Cancer Module score (see electronic Supplementary Table 1).

These values were calculated by dividing the total sample into different groups based on age and treatment status. The control study sample size (Nc_{total}) was 245, and the brain-tumor sample size (N_{total}) was 165 if all respondents completed the PedsQL Cancer Module scale. The control and study populations were divided into groups (Nc_{ij} and N_{ij}) separated by treatment status (on-treatment, off-treatment ≤12 months, or off-treatment >12 months; $i = 1$–3) and by age (2–4, 5–7, 8–12, or 13–18 years; $j = 1$–4). The weighted means [31] were calculated as follows:

$$\text{Weighted mean}(\bar{X}) = \sum_{k=1}^{N_{total}} W_k X_k \Big/ \sum_{k=1}^{N_{total}} W_k.$$

$$\left(\text{The common mean} = \sum_{k=1}^{N_{total}} X_k / N_{total}\right).$$

$$W_k = \left(\frac{Nc_{ij}}{Nc_{total}}\right) \Big/ \left(\frac{N_{ij}}{N_{total}}\right).$$

where X_k was the PedsQL Cancer Module scale score of each respondent that belonged to treatment status i and age j; the weights for each respondent (W_k) were calculated from the ratio of the age and treatment status of the standard population, divided by the proportion of the age and treatment status in this study.

The weighted SDs were calculated using the same weight (W_k) as follows:

$$\text{Weighted SD} = \sqrt{\sum_{k=1}^{N_{total}} W_k (X_k - \bar{X})^2 \Big/ \left(\sum_{k=1}^{N_{total}} W_k - 1\right)}.$$

$$\left(\text{The common SD} = \sqrt{\sum_{k=1}^{N_{total}} (X_k - \bar{X})^2 / (N_{total} - 1)}.\right)$$

We compared the cancer-specific HRQOL using Welch's t test and calculated the effect size d from the difference between the two means divided by the pooled SD of both samples.

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Table 2 PedsQL Cancer Module scores of children with brain tumors ($N = 143$)

	Mean	SD	Min.	Max.	Alpha[a]	ICC[b]
Child report (n = 124)						
Pain and Hurt	90.4	17.6	0	100	0.62	0.20
Nausea	87.5	20.6	15.0	100	0.86	0.68
Procedural Anxiety	74.5	30.8	0	100	0.88	0.70
Treatment Anxiety	92.8	19.0	0	100	0.88	0.41
Worry	81.9	23.4	0	100	0.76	0.27
Cognitive Problems	73.6	22.4	0	100	0.78	0.44
Perceived Physical Appearance	73.8	26.3	0	100	0.71	0.28
Communication	68.5	29.9	0	100	0.77	0.45
Parent report (n = 143)						
Pain and Hurt	84.5	20.0	0	100	0.83	
Nausea	84.7	22.6	15.0	100	0.93	
Procedural Anxiety	59.8	35.4	0	100	0.96	
Treatment Anxiety	79.7	23.1	0	100	0.93	
Worry	78.3	22.3	0	100	0.86	
Cognitive Problems	66.0	23.8	0	100	0.89	
Perceived Physical Appearance	70.6	24.6	0	100	0.81	
Communication	59.5	29.6	0	100	0.89	

ICC intraclass correlation coefficient, *Max.* maximum, *Min.* minimum, *SD* standard deviation

[a] Cronbach's alpha coefficient

[b] ICC values for child and parent reports in the two-way mixed effects model ($n = 124$)

The agreement of the two modules was evaluated using paired t tests; the effect size d (the mean score difference divided by SD of the mean score difference) [28] designated as small (0.20), medium (0.50), and large (0.80) in magnitude and by the ICC calculated from a one-way random effects model [27].

Results

Sample characteristics

The median age of the children with brain tumors was 10.0 years (range: 2–18) (Table 1), and the sample was heterogeneous for tumor pathology. Most children presented with embryonal tumors, low-grade gliomas, and germ cell tumors. Median age at diagnosis was 6.0 years; 63 children (39.4 %) were still receiving treatment, while 97 (60.6 %) had completed treatment, and the interval from completion of treatment to the survey ranged from 0.1 to 13.3 years. Most children on treatment were younger than the children who had completed treatment.

With the exceptions noted below, no significant differences were observed between the characteristics of the children and their parents and those of the historical control (Table 1). The differences were as follows: The present study enrolled fewer children between the ages of 5 and 7 years and more between the ages of 13 and 18 years ($P = 0.069$, Chi-square test).

Scale descriptions

The child-reported scores were higher than parent-reported scores on all scales of the PedsQL Cancer Module and were internally consistent for all scales except for the Pain and Hurt scale (Cronbach's alpha coefficient = 0.62); parent-reported scores were internally consistent for all scales (Table 2). Agreement between the child and parent reports was good for the Nausea and Procedural Anxiety scales, moderate for the Treatment Anxiety, Cognitive Problems, and Communication scales, and fair for the Pain and Hurt, and Perceived Physical Appearance scales.

Cancer-specific HRQOL in children with brain tumors compared with the HRQOL of children with other cancers

We noted small but significant differences between the children's reports for Pain and Hurt ($d = 0.26$) and Nausea ($d = 0.23$) and the parents' reports for Nausea ($d = 0.28$), Procedural Anxiety ($d = -0.22$), and Treatment Anxiety ($d = -0.32$) (Table 3). The scores for Pain and Hurt and Nausea were higher for children with brain tumors than for children with other cancers, indicating better HRQOL. However, the scores for Procedural Anxiety and Treatment Anxiety were lower for children with brain tumors than for children with other cancers, indicating worse HRQOL. The direction of the effects was the same for the scales reported by parents and children.

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Table 3 Comparison of cancer-specific HRQOL in children with brain tumors and those with other cancers

	This study[a]		Tsuji et al. [25][b]			P[c]	Effect size d[d]
	Mean	SD	n	Mean	SD		
$N = 143$							
Child report ($n = 124$)							
Pain and Hurt	89.8	19.3	202	84.7	19.7	0.024	0.26
Nausea	88.0	20.0	199	83.0	24.0	0.044	0.23
Procedural Anxiety	72.5	32.8	203	72.9	31.0	0.910	−0.01
Treatment Anxiety	90.7	22.8	203	93.1	17.0	0.302	−0.12
Worry	81.0	25.8	202	76.6	25.9	0.140	0.17
Cognitive Problems	72.3	23.8	200	71.5	22.1	0.775	0.03
Perceived Physical Appearance	71.9	28.7	204	70.3	28.6	0.639	0.05
Communication	65.5	32.6	204	67.0	27.0	0.656	−0.05
Parent report ($n = 143$)							
Pain and Hurt	84.9	20.9	242	82.9	22.0	0.367	0.09
Nausea	87.0	20.8	233	80.5	25.7	0.008	0.28
Procedural Anxiety	55.7	36.6	242	63.2	31.8	0.043	−0.22
Treatment Anxiety	77.9	24.4	241	84.9	19.0	0.004	−0.32
Worry	79.0	23.6	242	81.4	21.9	0.334	−0.10
Cognitive Problems	65.8	24.9	243	69.4	21.6	0.151	−0.15
Perceived Physical Appearance	71.7	25.3	243	73.8	24.9	0.437	−0.08
Communication	60.1	31.1	241	62.2	25.4	0.496	−0.07

HRQOL health-related quality of life, *SD* standard deviation

[a] Means and SDs of the PedsQL Cancer Module score in children with brain tumors adjusted for age and treatment status to subjects reported by Tsuji et al. [25]

[b] Previously reported data in children with the other cancers

[c] P value from the Welch t test

[d] Effect size d defined by Cohen [28] is the difference between two means divided by a pooled SD with two samples. A positive value indicates that children with brain tumors have higher HRQOL scores compared with children with other cancers

Agreement between the PedsQL cancer and the PedsQL Brain Tumor Modules of the PedsQL

Children and parents reported higher Pain and Hurt scores ($d = 0.29$, $P = 0.001$ and $d = 0.22$, $P = 0.010$, respectively) on the Cancer than on the Brain Tumor Module (Table 4). Children reported higher Procedural Anxiety ($d = 0.31$, $P = 0.001$) and Cognitive Problems scores ($d = 0.28$, $P = 0.003$) on the Cancer Module. The agreement between the PedsQL Cancer and the PedsQL Brain Tumor Modules was very high (ICC > 0.80) except for the Pain and Hurt scale for the child report where the agreement was moderate (ICC = 0.43). The agreement according to treatment status is shown in Supplementary Table 2.

Discussion

We report here that children with brain tumors perceive their HRQOL differently from children with other cancers. Several aspects of HRQOL were more difficult (for example, procedural and treatment anxiety) for patients with brain tumors, while other aspects (nausea, pain and hurt) were less difficult, and a number of factors may be responsible for these differences. In particular, the brain is the center of multiple functions. The brain integrates the information received from, and coordinates the physical and mental activity of, the whole body. Thus, the unique HRQOL of children with brain tumors likely reflects the vast complexity of brain function. Knowledge of these differences should help medical practitioners design-specific support and care strategies for these children.

A total of 29 % of children in this study suffered from embryonal tumors (mainly medulloblastomas), and treatment for these tumors requires surgery, radiation, and chemotherapy [32, 33]. The main treatments for children with germ cell tumors (mainly germinomas) include surgery, radiation, and chemotherapy [34], with chemotherapy representing the main treatment for children with leukemia (controls). Each treatment method will affect a child's HRQOL differently.

Table 4 Comparison of cancer-specific HRQOL using the PedsQL cancer and PedsQL Brain Tumor Modules

	n	Dif.[a]	95 % CI of the Dif.		*P*[b]	Effect size *d*[c]	ICC (5–18 years)[d]	ICC (2–18 years)[e]
			Lower	Upper				
$N = 143$								
Child report ($n = 124$)								
Pain and Hurt	124	5.41	2.12	8.70	0.001	0.29	0.43	–
Nausea	124	0.91	−0.91	2.72	0.325	0.09	0.88	–
Procedural Anxiety	123[f]	4.34	1.80	6.87	0.001	0.31	0.89	–
Worry	124	1.95	−0.39	4.30	0.102	0.15	0.84	–
Cognitive Problems	124	3.64	1.29	5.99	0.003	0.28	0.81	–
Parent report ($n = 143$)								
Pain and Hurt	143	2.50	0.60	4.40	0.010	0.22	0.82	0.91
Nausea	143	0.59	−1.20	2.39	0.515	0.05	0.91	0.89
Procedural Anxiety	142[f]	2.14	−0.77	5.05	0.148	0.12	0.88	0.87
Worry	143	1.46	−0.20	3.11	0.084	0.15	0.90	0.90
Cognitive Problems	124[g]	−0.99	−2.89	0.91	0.304	−0.09	0.89	–

CI confidence interval, *Dif.* difference, *HRQOL* health-related quality of life, *ICC* intraclass correlation coefficients, *PedsQL* pediatric quality of life inventory, *SD* standard deviation

[a] Mean score differences (PedsQL Cancer Module—PedsQL Brain Tumor Module). A positive value indicates that participants (children with brain tumors or parents of children with brain tumors) have higher scores in the PedsQL Cancer Module (fewer problems) than in the PedsQL Brain Tumor Module

[b] *P* value from the paired *t* test

[c] Effect size *d* defined by Cohen [28] is the mean score difference divided by SD of the mean score difference. A positive value indicates that participants (children with brain tumors or parents of children with brain tumors) scored higher in the PedsQL Cancer Module (fewer problems) than the PedsQL Brain Tumor Module

[d] ICC values for the PedsQL Cancer Module and the PedsQL Brain Tumor Module in the one-way random effects model among children aged 5–18 years

[e] ICC values for the PedsQL Cancer Module and the PedsQL Brain Tumor Module in the one-way random effects model among children aged 2–18 years

[f] Missing data for the Brain Tumor Module ($n = 1$) were excluded

[g] The PedsQL Brain Tumor Module parent report for toddlers (ages 2–4) does not include the Cognitive Problems scale

Children with brain tumors reported less difficulty with pain and hurt than children with other cancers; however, we believe it unlikely that these children actually experienced less pain, as here and in a previous study [17], parents reported similar difficulty with pain and hurt irrespective of cancer type. Children with brain tumors reported pain and hurt more frequently than children with lymphoma at a similar frequency to children with leukemia and less frequently than children with solid tumors [4]. These inconsistencies may arise due to scale characteristics. The agreement between Pain and Hurt scores in the Cancer and Brain Tumor Modules was moderate, while the agreement on other scales was high. These findings suggest that the Pain and Hurt scale of the PedsQL Cancer Module may not consider problems for children with brain tumors compared with the Brain Tumor Module.

The Pain and Hurt scale of the Cancer Module asks about generalized body pain but does not localize the pain. For example, "I ache or hurt in my joints and/or muscles," versus "I hurt a lot." Further, the Brain Tumor Module measures two items present in the Cancer Module and, uniquely, "I get headaches." Thus, the Brain Tumor Module includes a question about headaches, which are frequent in patients and survivors of brain tumors [35]. Headache is the most frequently reported initial symptom of pediatric brain tumors in children aged ≥2 years and may be interpreted with particular meaning for these children [36]. Headache would remind the children and parents of the first brain tumor and induce worry about a relapse. Such headaches cause physical distress and psychosocial concern. Therefore, we prefer to use the Brain Tumor to the Cancer Module to measure disease-specific HRQOL for these children.

Children with brain tumors and their parents reported less difficulty with nausea than children with other cancers. Causes of nausea may include side effects of chemotherapy, radiation sickness, postoperative reactions, tumors close to the area postrema, intracranial hypertension, gastrointestinal pathology, and anxiety [37, 38]. Here, at least 1 month had passed since diagnosis, and factors such as

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postoperative reaction, brain-tumor activity, and intracranial hypertension would have been controlled, resulting in less difficulty with nausea [39, 40].

Patients may experience strong nausea and vomiting at the onset of brain tumors as well as in the perioperative period; therefore, pediatric patients may evaluate their experience with treatment-induced nausea and vomiting as less trying than that experienced perioperatively. In contrast, children with ALL (control group majority) are treated at the first remission-induction phase using moderately emetogenic chemotherapy (i.e., vincristine, daunorubicin, L-asparaginase) [41], and severe emetogenic chemotherapy (i.e., cyclophosphamide, ifosfamide) is added during the intensification phase. Treatment type and course will affect a child's experience, so a longitudinal study will be required to assess how the experience of children with brain tumors changes after diagnosis and treatment.

Parents of children with brain tumors reported more procedural and treatment anxiety for their children than did the parents of children with other cancers. The PedsQL Cancer Module evaluates children's and parents' perception of a child's anxiety about needle sticks, blood tests, seeing a doctor, and hospitalization, which relate to trauma and stressor-related symptoms that are classified as anxiety disorders. Perceived life threat and treatment intensity are directly associated with posttraumatic stress disorder [42]. We assume that intensive symptoms and the treatment of pediatric brain tumors increase anxiety.

Our findings here of increased anxiety in children with brain tumors differ from those of a previous study conducted in the United States [17]. Although we cannot explain the reason for this discrepancy, pediatric oncology practice differs between the United States and Japan [43], and patients in Japan may not be fully informed of the diagnosis, which affects posttraumatic stress disorder [44]. Cognitive problems of children with brain tumors might also limit their understanding of disease and treatment course. Each child's psychological readiness for each stage of the diagnosis and treatment may be affected by the information provided and by the child's cognitive ability.

Several limitations of the present study warrant mention. First, the study and controls were heterogeneous and included various pathologies. All children in this study suffered central nervous system damage from invasion, compression, or hydrocephalus as well as from therapy. Further investigations of tumor types and treatment should reveal how HRQOL differs between children with brain tumors and those with other cancers.

Second, data obtained from children and parents were not completely equivalent; the ages of self-reporting children ranged between 5 and 18 years, whereas parental-reporting included children 2–18 years of age. Further, the varying degrees of patients' impairments prevented optimum accuracy of reporting [17]. However, the number of children participating in the present study (133) was similar to that of participating parents of children aged 5–18 years (140) because of assisted administration. Further, HRQOL reporting by children is not significantly influenced by the administration technique [24, 45].

Third, the PedsQL Cancer and Brain Tumor Modules employ different recall periods, as described above [15, 25]. This difference must be taken into account when interpreting data. Although the items on the Procedural Anxiety subscale are identical in both modules, children with brain tumors studied here reported less difficulty with procedural anxiety using the Cancer than with the Brain Tumor Module. The recall period may alter a child's perception of procedural anxiety. Further research is required to determine why children reported less anxiety over the past month than over the previous 7 days.

Fourth, our ability to generalize the data is limited. For example, at the CCAJ, several hundred families, including those not eligible to participate, were notified of this study; therefore, the true response rate is unknown. Families were excluded if doctors or social workers determined that the family found the child's condition too uncomfortable to discuss. Although the number of such excluded families was not recorded, this exclusion may have limited data collection.

Fifth, when comparing children with brain tumors to those with other cancers, certain parental characteristics could not be taken into account, as Tsuji et al. [25] did not report them. Parental reports might have been influenced by factors such as parental mental health, which may limit comparability. However, all child and parent characteristics reported here, except for age and tumor pathology, were similar.

Conclusion

Here, we found that children with brain tumors reported less difficulty with the categories of pain and hurt and nausea than children with other cancers that included mostly leukemia. Parents of the children with brain tumors reported more procedural and treatment anxiety. The information will help medical professionals and researchers to understand the influence of the disease and treatment on the HRQOL of children with brain tumors regardless of age and treatment status.

This study is the only comparison, to our knowledge, of the PedsQL Cancer and Brain Tumor Modules. The PedsQL Cancer Module compares cancer-specific HRQOL of children with brain tumors and those with other cancers. However, the PedsQL Brain Tumor Module is more

sensitive for brain-tumor-specific aspects of the HRQOL and should be used to assess HRQOL in children with brain tumors.

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SNP Association Mapping across the Extended Major Histocompatibility Complex and Risk of B-Cell Precursor Acute Lymphoblastic Leukemia in Children

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Abstract

The extended major histocompatibility complex (xMHC) is the most gene-dense region of the genome and harbors a disproportionately large number of genes involved in immune function. The postulated role of infection in the causation of childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) suggests that the xMHC may make an important contribution to the risk of this disease. We conducted association mapping across an approximately 4 megabase region of the xMHC using a validated panel of single nucleotide polymorphisms (SNPs) in childhood BCP-ALL cases (n=567) enrolled in the Northern California Childhood Leukemia Study (NCCLS) compared with population controls (n=892). Logistic regression analyses of 1,145 SNPs, adjusted for age, sex, and Hispanic ethnicity indicated potential associations between several SNPs and childhood BCP-ALL. After accounting for multiple comparisons, one of these included a statistically significant increased risk associated with rs9296068 (OR=1.40, 95% CI=1.19-1.66, corrected p=0.036), located in proximity to *HLA-DOA*. Sliding window haplotype analysis identified an additional locus located in the extended class I region in proximity to *TRIM27* tagged by a haplotype comprising rs1237485, rs3118361, and rs2032502 (corrected global p=0.046). Our findings suggest that susceptibility to childhood BCP-ALL is influenced by genetic variation within the xMHC and indicate at least two important regions for future evaluation.

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Introduction

Acute lymphoblastic leukemia (ALL) is a clonal disorder involving the dysregulated proliferation of genetically altered lymphoid progenitor cells that lack the ability for differentiation and maturation. In children, B-cell precursor (BCP) ALL is the most common ALL subtype and accounts for about 80% of childhood ALL cases in most economically developed countries. BCP-ALL, which demonstrates a unique age-incidence peak between 2 and 5 years of age, is widely suspected to be caused by environmental exposures, though these have yet to be definitively identified [1]. Foremost among these are thought to be factors such as the effect of timing of exposure to infectious agents leading to inappropriate immune responses, in conjunction with variants in genes of the immunological pathway and early lymphoid development [2].

The extended major histocompatibility complex (xMHC) region, spanning about 7.6 megabases (Mb) on the short arm of chromosome 6 (6p21.3), is densely populated with genes that are critical to both innate and adaptive immunity in humans [3]. The xMHC is divided into five sub-regions consisting of the extended class I region at the telomeric end, and successively the classical class I, III, and II clusters bounded by an extended class II region at the centromeric end. Historically, genetic association studies of the xMHC have focused on the classical human leukocyte antigen (HLA) genes of the class I (*HLA-A*, *B*, and *C*) and class II (*HLA-DP*, *DQ*, and *DR*) regions. These encode cell surface glycoproteins that selectively bind and present, in allele selective fashion, processed antigenic peptides to T lymphocytes that initiate T-cell responses. While *HLA* genes are among the most polymorphic in humans, they account for only a small proportion of over 250 expressed xMHC loci, which include genes encoding cytokines, complement factors, and various others involved in critical cellular processes.

Evidence of susceptibility associated with xMHC loci has been identified in several autoimmune, malignant and infectious diseases, including asthma, Hodgkin and non-Hodgkin lymphoma, hepatitis B and HIV infection and others [4,5,6,7]. Despite evidence of linkage between predisposition to retrovirus-induced leukemia and the murine MHC, attempts to identify associations between childhood ALL and classical HLA alleles have been inconsistent largely due to study design limitations [8,9,10,11,12]. Although these have been largely overcome by the application of high-resolution HLA molecular genotyping to carefully ascertained case-control series, strong and replicable associations have yet to emerge.

A previous analysis of MHC SNP data and imputed HLA class I and II alleles derived from a childhood ALL genome-wide association study (GWAS) suggested that MHC genetic variation is unlikely to be a major determinant of BCP-ALL [13]. Nonetheless, a modest SNP association noted in the HLA class II region, together with strong positive findings from the largest study to date utilizing directly typed HLA genotypes [9], suggested that further examination of the xMHC in childhood ALL was warranted in a well-defined case-control series. We report here the results of SNP association mapping across a 4 Mb stretch of the xMHC spanning all major class I, II, and III loci using a validated SNP panel in a large sample of non-Hispanic white and Hispanic BCP-ALL cases (n=567) and controls (n=892) enrolled in the Northern California Childhood Leukemia Study (NCCLS).

Materials and Methods

Ethics Statement

The study protocol was approved by the Institutional Review Boards of the University of California, Berkeley and all collaborating institutions (California Department of Public Health, University of California, Davis, University of California, San Francisco, Children's Hospital of Central California, Lucile Packard Children's Hospital, Children's Hospital and Research Center, Oakland, Kaiser Permanente, Roseville, Kaiser Permanente, Santa Clara, Kaiser Permanente, San Francisco, Kaiser Permanente, Oakland), and written informed consent was obtained from the parents or guardians on behalf of the children participants involved in this study. This study was conducted in accordance with the Declaration of Helsinki.

Study participants

The current study was conducted within the NCCLS, an ongoing case-control study of childhood leukemia. Beginning in 1995, newly diagnosed childhood leukemia cases were ascertained at the time of diagnosis from major pediatric hospitals in a 17-county San Francisco Bay Area study region, expanded in 1999 to 35 counties in Northern and Central California, USA. Comparison with the California Cancer Registry (1997-2003) showed that the NCCLS case ascertainment protocol has captured about 95% of children diagnosed with leukemia in the participating study hospitals. For each eligible case, statewide birth records maintained by the California Office of Vital Records were utilized to generate a list of randomly selected controls that matched the case on child's date of birth, sex, Hispanic ethnicity (a biological parent who is Hispanic), and maternal race. Information obtained through the birth certificates and commercially available searching tools were used to trace and enroll one or two matched controls for each case.

Cases and controls were considered eligible if they were under 15 years of age at date of diagnosis for cases (or corresponding reference date for controls), residents of the study region, had a biological parent who spoke either English or Spanish, and had no prior history of malignancy. Approximately 85% of eligible cases and 86% of eligible controls consented to participate [14]. A detailed description of control selection in the NCCLS is reported elsewhere [14,15].

In the current study, non-Hispanic white and Hispanic children with ALL and control children, recruited between 1995 and 2008 (study phases 1-3), were included in the analysis. These are the two largest racial/ethnic groups which together comprise about 85% of enrolled subjects. Other ethnic groups were excluded due to the small number of subjects. Children were classified as Hispanic if at least one biological parent self-identified as Hispanic. Children were assigned to the non-Hispanic white group if both biological parents self-identified as non-Hispanic white. In a previous NCCLS analysis, genetic admixture was assessed using a series of 80 ancestry informative markers for a subset of the cases and controls [16], and estimates of genetic ancestry (percent of European, Amerindian, and African ancestry) were determined. Comparison of these estimates between cases and matched controls showed no significant differences [16]. For the current study, a total of six hundred and eighty-eight ALL cases and 1,012 controls were considered, of which 635 cases (92.3%) and 915 controls (90.4%) had a DNA sample available for genotyping.

DNA samples

Buccal cells as a source of DNA were obtained from case and control children using cytobrushes by trained interviewers.

Cytobrushes were processed within 48 hours of collection by heating in the presence of 0.5N NaOH. Isolated DNA was later re-purified either manually using Gentra Puregene reagents (QIAGEN, USA, Valencia, CA) or an automated organic DNA extraction protocol (AutoGen, Holliston, MA). Whole genome amplification (WGA) was performed using GenomePlex reagents (Rubicon Genomics, Ann Arbor, MI) according to the manufacturer's protocol. WGA products were cleaned with a Montage PCR9 filter plate (Millipore, Billerica, MA). When buccal cytobrush DNA was inadequate or not available (26.6% of subjects), DNA was isolated from dried bloodspots collected at birth and archived at -20°C by the Genetic Disease Screening Program of the California Department of Public Health. After extraction using the QIAamp DNA Mini Kit (QIAGEN, USA, Valencia, CA), DNA samples were whole-genome amplified using REPLI-g reagents (QIAGEN, USA, Valencia, CA). Regardless of source, DNA specimens were quantified using human-specific Alu-PCR to confirm a minimum level of amplifiable human DNA [17].

MHC genotyping

Genotyping was conducted using the Illumina MHC Mapping Panel (Illumina Inc., San Diego, CA) which comprises 1,293 SNPs spanning an approximately 4 Mb region of the xMHC bounded by the tripartite motif containing protein 27 (*TRIM27*) and motilin (*MLN*) genes at the telomeric and centromeric ends, respectively (NCBI Build 36). There is an average 3.8 kilobase (kb) spacing between each SNP, covering all major regions of the xMHC, including the classical class I, II and III regions, the extended class II region and part of the extended class I region. The panel set was designed with a strong emphasis on haplotype tagging SNPs which are highly informative of SNPs in strong linkage disequilibrium (LD). The chance of detecting an association is significantly influenced by the ability of a SNP or combination of SNPs to adequately represent the haplotypic diversity of the region. Genotyping was performed utilizing the robust Golden-Gate technology in a 96-well format on a 1,536 Sentrix Array Matrix [18]. It was shown previously in NCCLS subjects that when analyzed using Golden-Gate genotyping, buccal cell WGA DNA yielded genotypes that are highly concordant with those from genomic DNA from peripheral blood [19].

Genotyping was conducted on 1,550 unique DNA samples (635 cases and 915 controls), in addition to 10 sets of *Centre d'Etude du Polymorphisme Humain* (CEPH) family trios and duplicates of 10% of study samples. For quality control purposes, 113 SNPs that successfully genotyped in less than 90% of samples were excluded, as well as 30 SNPs that deviated from Hardy Weinberg equilibrium ($p<0.01$) in both non-Hispanic white and Hispanic controls, and 5 additional SNPs with a minor allele frequency of less than 0.01 in both non-Hispanic white and Hispanic controls.

Quality control metrics applied to the 1,550 samples also resulted in the exclusion of 17 samples (1.1%) with less than 95% overall genotyping success rate and 20 samples (1.3%) that showed questionable concordance between reported gender and gender prediction by the Illumina platform. There was 99.6% concordance of successfully genotyped SNPs in the duplicate series and a 0.2% Mendelian error rate was observed in the CEPH family trios. Application of these quality control criteria and a focus on BCP-ALL (54 T-cell or mixed lineage ALL cases excluded) resulted in the analysis of 1,145 SNPs in a total of 567 BCP-ALL cases and 892 controls (Table 1). Data are available on request in accordance with the policies and procedures of the NCCLS.

Table 1. Characteristics of childhood BCP-ALL cases and controls.

	Cases		Controls	
	n	%	n	%
Total	567	100	892	100
Sex				
Male	298	52.6	495	55.5
Female	269	47.4	397	44.5
Age (years)				
0-1	51	9.0	111	12.4
2-5	340	60.0	460	51.6
6-10	114	20.1	210	23.5
11-14	62	10.9	111	12.4
Self-reported race/ethnicity				
White, non-Hispanic	241	42.5	426	47.8
Hispanic	326	57.5	466	52.2
BCP-ALL subtype[a]				
cALL[b]	309	49.8	NA	NA
Non-cALL	258	41.5	NA	NA
High-hyperdiploid	178	28.7	NA	NA
TEL-AML1	96	15.5	NA	NA
Normal karyotype	58	9.3	NA	NA

Abbreviations: BCP-ALL, B-cell precursor acute lymphoblastic leukemia; cALL, common ALL; NA, not applicable

[a] Categories of ALL subtypes are not mutually exclusive. Percentages do not equal to 100. Cytogenetic data were available for 87% of patients.

[b] cALL is defined as CD10+ and CD19+ ALL diagnosed between age 2-5 years.

For a large subset of the BCP-ALL cases (87%), data on hyperdiploidy and *TEL-AML1* chromosomal translocation were available as described in detail previously [20]. Subtypes of the cases included 309 common ALL (cALL, defined as CD10+ and CD19+ ALL aged 2 to 5 years), 178 high-hyperdiploid (51-67 chromosomes), 96 positive for the *TEL-AML1* chromosomal translocation, and 58 BCP-ALL with normal karyotypes. These non-mutually exclusive subtype groupings were used to examine potential subtype-specific effects for the final set of associated SNPs.

Statistical analysis

Data analysis included a two-stage approach. First, we examined the contribution of 1,145 xMHC SNPs individually. We used logistic regression to calculate the odds ratio (OR) and 95% confidence intervals (CI) for each SNP adjusting for child's age, sex, and race/ethnicity (i.e. non-Hispanic white or Hispanic). Various genetic models of inheritance were considered including log-additive, dominant, and recessive models, in addition to an evaluation of the dominance deviation from additivity. SNPs showing a nominal p-value of less than 0.01 in any of these analyses were considered potentially associated with childhood ALL and were subject to further analysis. Multivariable logistic regression was used to evaluate the independence of effect of multiple potentially associated SNPs within a region on childhood ALL risk. Stratified analyses by age (0-5 and 6-14 years) and gender were considered in sub-analyses of the data. To account for multiple comparisons in the presence of LD between SNPs, we calculated adjusted p-values based on 10,000 permutations of case-control status on 1,145 SNPs and considered adjusted p-values below a family-wise type I error rate threshold of 0.05 to be statistically significant.

Second, we conducted three-SNP sliding window haplotype analyses across candidate regions selected by the location of SNPs that showed nominal p-values of less than 0.01. This resulted in 3 broad regions (Figure 1) for the haplotype analysis: 1) a region bound by rs381808 and rs3117330 referred to as region A (~364 kb region, extended class I); 2) a region bound by rs1264419 and rs3828886 referred to as region B (~864 kb region, class I-class III); and 3) a region bound by rs516535 and rs210134 referred to as region C (~598 kb region, class II-extended class II). In total, the haplotype sliding window analysis was performed on 395 genotyped SNPs (393 3-SNP haplotypes) located within these 3 regions. Haplotypes were predicted and reconstructed for each individual and frequencies estimated using the expectation-maximization algorithm based on unphased genotype data. Haplotypes with a frequency of less than 0.01 were grouped into one category. For each three-SNP sliding window, a global likelihood ratio test of association was performed to test the null hypothesis of no effect of any haplotype at that position. The permutation method was used to adjust for multiple comparisons for the 393 haplotype windows tested. Haplotype-specific effects were evaluated by modeling individual haplotype probabilities in a logistic regression assuming a log-additive effect of the haplotype and adjusting for age, sex, and race/ethnicity. Analyses were conducted using PLINK, UNPHASED version 3.1.4, and Haploview [21,22,23].

Results

Case and control distributions for sex, age, and race/ethnicity were similar, as expected from the matched design of the NCCLS (Table 1). Hispanics comprised about 58% of cases and 52% of controls.

Analysis of 1,145 SNPs in childhood BCP-ALL, assuming a log-additive genetic model of inheritance, showed a quantile-quantile (Q-Q) plot of the expected versus observed $-\log_{10}$ p-value distribution that suggested little evidence of inflation in results caused by systematic error (Figure S1). Twenty SNPs were associated with a nominal p-value of less than 0.01 (log-additive, genotypic, dominant, and/or recessive genetic models), many of which were in LD (Figure 1 and Figure S2). These SNPs (Figure 1) appeared to cluster within three specific regions (designated A, B, and C) which served as the focus of the haplotype analysis. SNPs showing the strongest evidence of association based on p-value within each of the three regions included rs7747023 (OR=0.73, 95% CI=0.60-0.89, $p=1.7\times10^{-3}$) of region A, rs3130785 (OR=1.45, 95% CI=1.16-1.82, $p=1.3\times10^{-3}$) of region B, and rs9296068 (OR=1.37, 95% CI=1.17-1.61, $p=1.2\times10^{-4}$) of region C. Multivariable analyses evaluating the independence of associations between the 20 SNPs on BCP-ALL risk (Table S1) resulted in 6 SNPs that maintained low p-values and minimally attenuated risk estimates (Table 2). Stratified analysis showed no remarkable gender- or age-specific associations (Figure S3). The final multivariable model including all 6 SNPs and correcting for multiple comparisons showed a statistically significant association between rs9296068 and BCP-ALL risk (OR=1.40, 95% CI=1.19-1.66, corrected p=0.036).

Further examination of SNP rs9296068 (Figure 2) showed little evidence of heterogeneity in effect between non-Hispanic white and Hispanic children (p=0.503), males and females (p=0.356), and children aged zero to five and aged six to fourteen (p=0.906). The risk estimates appeared consistently elevated for the two main cytogenetic subtypes (i.e. *TEL-AML1*-positive and high hypderdiploidy), but not for normal karyotype BCP-ALL (Figure 2, OR=1.26, 95% CI=0.83-1.91).

We performed a 3-SNP sliding window haplotype analysis that included 395 genotyped SNPs across each of the 3 candidate regions (regions A, B, and C) identified by the single SNP analysis. After adjusting for multiple comparisons, a statistically significant association was found for the rs1237485-rs3118361-rs2032502 haplotype (nominal global $p=3.2\times10^{-4}$; corrected p=0.046) in region A (Table 3 and Figure 3A). Specifically, haplotype G-A-G was associated with an increased risk of BCP-ALL compared to other haplotypes combined (OR=2.18, 95% CI=1.41-3.38) (Table 3). In multivariable analysis adjusting for the nearby region A SNP, rs7747023 (described above in the individual SNP analysis), evidence of association for this haplotype remained strong (nominal global $p=2.5\times10^{-3}$), while the effect for rs7747023 appeared to be attenuated. When rs7747023 was included in the haplotype, the global test for the 4-SNP haplotype (rs1237485-rs3118361-rs2032502-rs7747023) yielded stronger evidence of an association (nominal global $p=2.7\times10^{-5}$). Stratified analyses by race/ethnicity showed similar results in non-Hispanic white and Hispanic children.

Finally, haplotype rs423639-rs7754316-rs9296068 (Table 3 and Figure 3B) of region C was found to be statistically significant (nominal global $p=9.2\times10^{-5}$; corrected p=0.014), a locus also identified through the individual SNP analysis of rs9296068. Previously, we reported an association between childhood ALL risk and the *DP1* supertype of *HLA-DPB1* (comprised of *HLA-DPB1* alleles *01:01*, *05:01*, *50: 01*) [12], a class II gene located about 55 kb from the associated rs9296068 SNP. The two loci showed weak correlation ($r^2<0.1$) and the analysis adjusting for carriers of the *DP1* supertype indicated an independent effect for rs9296068 (OR=1.37, 95% CI=1.16-1.63, $p=3.0\times10^{-4}$).

Discussion

In this study, we conducted a SNP-association analysis of childhood BCP-ALL compared with controls across a 4 Mb

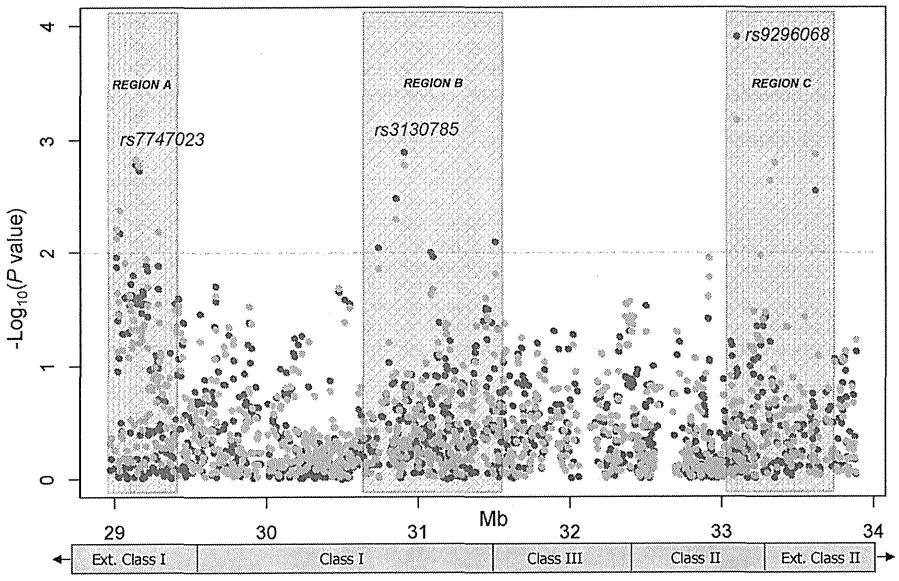

Figure 1. Analysis of 1,145 SNPs across a 4 Mb region of the extended major histocompatibility complex and risk of childhood BCP-ALL. Presented are -$\log_{10}$(p-values) resulting from the logistic regression analysis assuming log-additive (navy blue) and dominant (light blue) genetic models of inheritance and adjusting for child's age, sex, and race/ethnicity. Results plotted above the dotted line represent nominal p-values of less than 0.01. Analyses evaluating the recessive and genotypic genetic models were also performed (not plotted) resulting in five additional SNPs with a nominal p-value of less than 0.01 which were also located within one of the three designated regions (Regions A-C). A total of 20 SNPs with a p-value below this threshold were considered in further analyses.

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stretch of the xMHC in an attempt to pinpoint regions of potential involvement in susceptibility. The xMHC is a potentially strong candidate region for a role in genetic susceptibility to childhood ALL, a disease whose causation has been attributed to an inappropriate immune response to post-natal infection [2,24,25]. Using a validated panel of greater than 1,100 SNPs designed to capture the genetic diversity of this complex genomic region, we identified two loci associated with childhood BCP-ALL risk. After correction for multiple testing, we found a statistically significant increased risk associated with the minor allele of rs9296068 in proximity to the *HLA-DOA* gene. A second independently associated locus, represented by haplotypes comprised of SNPs rs1237485, rs3118361, and rs2032502, was identified using a haplotype sliding window analysis and is located in the extended class I region in proximity to the *TRIM27* gene.

The rs9296068 SNP is located in the 5' untranslated region of the *HLA-DOA* gene about 11.3 kb from the first exon and resides within a region that is predicted to have promoter function [26,27]. *HLA-DOA* encodes the alpha subunit of the HLA-DO heterodimer and is selectively expressed in B-cells and thymic medullary epithelium [28]. HLA-DO interacts with HLA-DM to regulate peptide loading onto MHC class II molecules in a pH-dependent manner. While HLA-DM facilitates peptide binding by catalyzing the exchange between low and high affinity peptides, HLA-DO impedes this function by reducing class II-mediated presentation in general, and has the ability to skew the presented antigenic peptide repertoire in B cells [29]. Thus, a balanced expression between HLA-DM and DO is critical in controlling antigen presentation in B-cells.

Recently, HLA-DOA has been implicated in other disease association studies such as type 1 diabetes and chronic lymphocytic leukemia survival [30,31], and interestingly, the same rs9296068 SNP was reported in a multistage MHC association mapping study of pediatric liver transplant rejection [26]. Functional validation showed a nearly 3-fold higher intra-graft B-lymphocyte content in rejecting liver grafts among carriers of the risk allele compared to non-carrier rejecters. This

Table 2. Association between xMHC genetic variants and BCP-ALL in children.

				Frequency[a]		Single SNP			Mutually adjusted			
SNP	Position	Region of xMHC	Minor allele	Cases	Controls	OR	95% CI[b]	p-value	OR	95% CI[b]	p-value	p-value $_{(FWE)}$[c]
rs7747023	29133659	Extended class I	G	0.17	0.21	0.73	(0.60-0.89)	$1.7x10^{-3}$	0.72	(0.59-0.88)	$1.4x10^{-3}$	0.518
rs3130785	30904717	Class I	A	0.14	0.11	1.45	(1.16-1.82)	$1.3x10^{-3}$	1.37	(1.09-1.74)	$7.9x10^{-3}$	0.973
rs1632856	31079715	Class I	A	0.25	0.29	0.80	(0.68-0.95)	$9.9x10^{-3}$	0.79	(0.66-0.94)	$8.6x10^{-3}$	0.898
rs2524279	31500885	Class I	G	0.11	0.15	0.73	(0.58-0.92)	$7.9x10^{-3}$	0.70	(0.55-0.89)	$3.0x10^{-3}$	0.749
rs9296068	33096673	Class II	C	0.42	0.36	1.37	(1.17-1.61)	$1.2x10^{-4}$	1.40	(1.19-1.66)	$5.7x10^{-5}$	0.036
rs213203[d]	33346382	Extended class II	A	0.47	0.49	0.68	(0.55-0.84)	$3.6x10^{-4}$	0.69	(0.55-0.86)	$7.4x10^{-4}$	0.347

Abbreviations: CI, confidence interval; FWE, family-wise type I error; OR, odds ratio; SNP, single nucleotide polymorphism; xMHC, extended major histocompatibility complex

[a] Frequency of the minor allele in case and control subjects

[b] ORs and 95% CI for each SNP in the single SNP analysis were derived using logistic regression assuming a log-additive genetic model of inheritance and adjusting for child's age, sex, and race/ethnicity (non-Hispanic white versus Hispanic). The mutually adjusted analysis included additional adjustment for the effects of all other SNPs in the table.

[c] Adjustment for multiple testing was performed with 10,000 permutations of case-control status on 1,145 SNPs and using FWE rate of 0.05. An adjusted p-value of less *than 0.05 was considered statistically significant.*

[d] Evaluation of the genetic model of inheritance indicated a significant deviation from the log-additive model with an effect associated with heterozygotes. ORs and 95% CI were estimated for heterozygous genotypes compared to homozygous genotypes.

SNP was also associated in a recent GWAS of rheumatoid arthritis, but its independence from the known *HLA-DRB1* effect was not described [32]. Further biological relevance of this SNP locus is supported by publicly available expression quantitative trait loci data, with one source indicating an rs9296068 allelic- dependent association with *HLA-DOA* gene expression in lymphoblastoid cell lines [26,33], and another showing associations with *HLA-DPB1* gene expression in purified B-cells and monocytes [34].

Previous examinations of the xMHC in childhood ALL have mostly been candidate gene studies that focused on the classical HLA genes (i.e. *HLA-A*, *-B*, and *-C*, and *HLA-DP*, *-DQ*, and *-DR*). The most consistent evidence of an association has been for HLA class II loci, including *HLA-DPB1* [10,12] and *HLA-DR* [8,35], genes relatively close in proximity to rs9296068 and *HLA-DOA*. However, due to the lack of genetic characterization of the surrounding regions in these studies, it could not be unambiguously determined whether those associations indicated a causal link with the HLA gene or whether the associations were an effect of LD with an adjacent causal locus. The availability of directly genotyped *HLA-DPB1* data allowed us to confirm that the rs9296068 association of the class II region is independent of the *HLA-DPB1* allelic associations previously reported in the CCLS and elsewhere [10,12]. We were unable to confirm this for *HLA-DQ* and *HLA-DR*, but the presence of major recombination activity and the weak correlation between rs9296068 and SNPs immediately upstream make it unlikely that the association originates from the DQ/DR loci [36].

Authors of a recent report using data extracted from a prior GWAS analysis concluded no substantive support for a major role of MHC genetic variation on childhood BCP-ALL risk [13]. Among the results, the strongest single SNP association was observed for rs3135034 which exhibited a nominal p-value of 0.0017, but was not statistically significant after correction for multiple testing. SNP rs3135034 is located about 20 kb downstream of *HLA-DOA*, and only 37 kb from rs9296068 in an intergenic region also in proximity to the bromodomain containing 2 (*BRD2*) gene. In our data, rs3135034 is weakly correlated (r^2<0.1) with rs9296068 in both non-Hispanic whites and Hispanics and showed no association with childhood BCP-ALL. However, rs9296068 and rs3135034 flank a well-characterized strong meiotic recombination hotspot, *DNA3* [37], and it has been noted that etiological variants within a recombination hot spot may be impossible to identify using standard association strategies [38]. This suggests that the identification of these two SNPs in close physical proximity in two independent studies of MHC association with childhood ALL could indicate a true association with *HLA-DOA*, partially masked by *DNA3*.

Using a haplotype sliding window analysis, we identified a second independently associated locus which is localized to the extended class I region and is represented by a haplotype comprised of SNPs rs1237485, rs3118361, and rs2032502. This haplotype maps to the 5' untranslated region of the *TRIM27* gene about 2.5 kb from the coding region and is greater than 1Mb from the nearest classical HLA locus (*HLA-A*). TRIM27 (also known as Ret finger protein, RFP) belongs to an expanding family of proteins that are characterized by a tripartite motif comprising Really Interesting New Gene (RING) and B-box zinc-binding domains, and a variable coiled-coil region [39]. They participate in a variety of critical biological processes including cell growth, tumor suppression, DNA damage signaling, senescence, apoptosis, stem cell differentiation, and immune response to infections. Recent evidence demonstrated that TRIM27 (and other TRIM proteins) may contribute to a repertoire of pathways through its function as both a small ubiquitin-like modifier (SUMO) protein and ubiquitin E3 ligase important in post-translational modification [40,41]. The p53 tumor suppressor and its principal antagonist murine double minute 2 (Mdm2) oncogene are among the several substrates of TRIM27 SUMO and ubiquitin E3 ligase

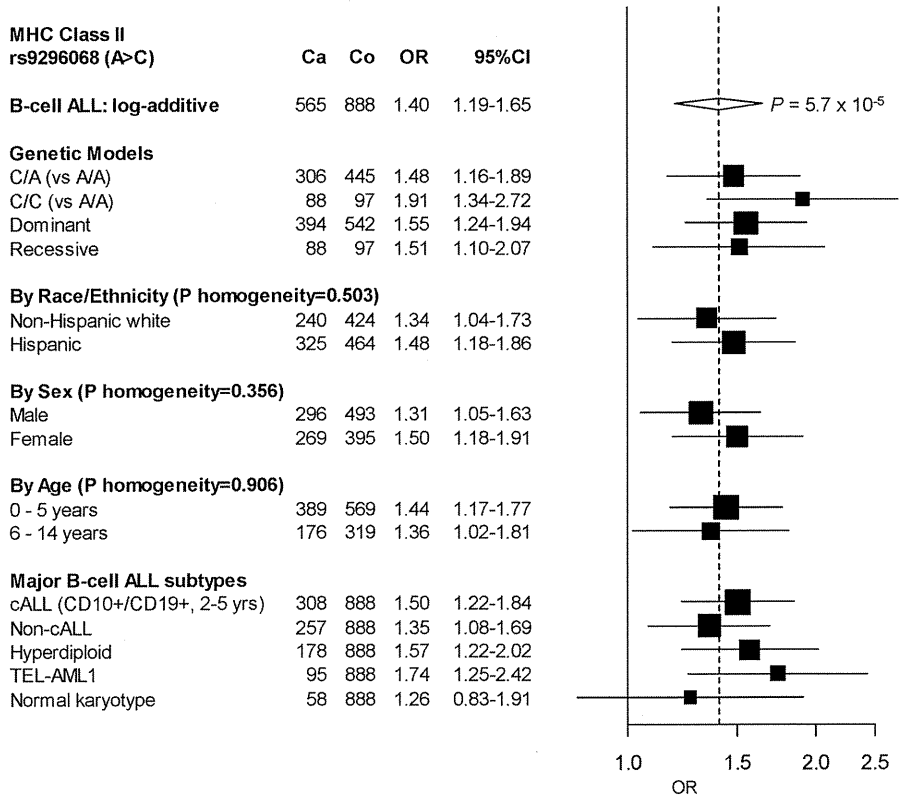

Figure 2. Stratified analysis of childhood BCP-ALL and the SNP rs9296068 by race/ethnicity, sex, and age group, and subgroup analyses by major subtypes. Odds ratios (ORs, represented by boxes with the area of each box inversely proportional to the variance of the estimate) and 95% CI (error bars) were derived using logistic regression assuming a log-additive genetic model and adjusting for rs7747023, rs3130785, rs1632856, rs2524279, and rs213203 (other potentially associated SNPs presented in Table 1) and additionally for child's age, sex, and race/ethnicity based on the stratification variable. The dashed vertical line represents the OR of the SNP in the analysis of BCP-ALL among all subjects and the width of the diamond is the corresponding 95% CI. $P_{homogeneity}$ was on the basis of the Cochran's Q test statistic. Abbreviations: Ca, number of case; cALL, common acute lymphoblastic leukemia; Co, number of controls.

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activity. With respect to immune regulation, TRIM27 is thought to down-regulate the immune response at multiple levels, including inhibition of toll-like receptor activation of nuclear factor-kappaB (NF-κβ) and interferon regulatory factor 3 (IRF3) [42], and the ability to negatively regulate CD4+ T cells through inhibitory effects on KCa3.1 (calcium-activated potassium channel) protein activity [43,44].

We did not impute genotypes for additional xMHC SNP loci or classical HLA alleles because certain features of the current study made it suboptimal for implementation of imputation including, 1) uncertainty in the use of currently available reference panels for imputation in a recently admixed population [45,46], 2) a focus on the xMHC, a region of complex LD that shows varying degrees of heterogeneity even

Table 3. Results for two associated regions based on a 3-SNP haplotype sliding window analysis of BCP-ALL cases and controls.

	Frequency		Compare to reference haplotype		Compared to all other haplotypes			
Haplotype	Cases (%)	Controls (%)	OR	95% CI[a]	OR	95% CI[a]	p-value	p-value$_{(FWE)}$[b]
Region A								
rs1237485-rs3118361-rs2032502[c]								
A-A-G	0.06	0.06	1.03	(0.72-1.48)	1.16	(0.82-1.62)	0.406	
A-G-A	0.10	0.13	0.84	(0.65-1.08)	0.74	(0.58-0.94)	0.015	
A-G-G	0.37	0.33	1.22	(1.02-1.45)	1.14	(0.97-1.34)	0.109	
G-A-G	0.06	0.03	2.44	(1.54-3.88)	2.18	(1.41-3.38)	2.9×10^{-4}	
G-G-G	0.41	0.45	*1.00*	*Ref*	0.89	(0.76-1.04)	0.136	
						Global p-value[d]	3.2×10^{-4}	0.046
Region C								
rs423639-rs7754316-rs9296068[c]								
A-A-A	0.07	0.08	0.82	(0.61-1.11)	0.93	(0.69-1.23)	0.576	
G-A-A	0.27	0.32	0.77	(0.63-0.93)	0.80	(0.67-0.95)	9.1×10^{-3}	
G-A-C	0.39	0.34	*1.00*	*Ref*	1.25	(1.07-1.46)	6.0×10^{-3}	
G-G-A	0.24	0.25	0.82	(0.67-1.00)	0.91	(0.76-1.10)	0.331	
G-G-C	0.04	0.02	1.98	(1.08-3.63)	2.47	(1.38-4.43)	1.7×10^{-3}	
						Global p-value[d]	9.2×10^{-5}	0.014

Abbreviations: CI, confidence interval; FWE, family-wise type I error; OR, odds ratio; SNP, single nucleotide polymorphism

[a] ORs and 95% CI for each haplotype were derived using logistic regression modeling haplotype probabilities and adjusting for child's age, sex, and race/ethnicity (non-Hispanic white versus Hispanic).

[b] Adjustment for multiple testing was performed with 10,000 permutations of case-control status on 393 haplotype windows and using FWE rate of 0.05. An adjusted p-value of less than 0.05 was considered statistically significant.

[c] The associated region A haplotype is located in the extended class I region and the SNPs are at chromosomal positions 29,002,323 (rs1237485), 29,006,266 (rs3118361), and 29,009,544 (rs2032502). The associated region C haplotype is located in the class II region and the SNPs are chromosomal positions 33,095,752 (rs423639), 33,095,976 (rs7754316), and 33,096,673 (rs9296068).

[d] Global p-values were derived based on a likelihood ratio test of association based on the null hypothesis of no effect of any haplotype at that position.

across sub-strata of individuals of European descent [47,48], and 3) a sample size comparably large for a study of a rare disease, but not statistically robust to a multiple comparisons burden that would be elevated by close to 10-fold with the additional loci. Thus, it is possible that associations were missed due to limited SNP coverage in certain regions.

The associations reported from these analyses were not identified in the previous GWAS [49,50,51,52,53]. Notably, our study was performed on a sample size comparable to that of the previous GWAS of childhood ALL, but with a substantially smaller multiple comparisons burden on statistical power due to its focus only on the xMHC. However, we acknowledge that statistical power may have been affected by combining non-Hispanic white and Hispanic children for the analysis. The success of the association mapping approach is highly dependent on the degree of LD between the genotyped SNP and the causal locus. A loss in precision would be expected if this LD between the SNP and causal locus differed across populations included in the analysis [54]. While described as a limitation, an advantage of our approach is that the detectable associations would likely only be those that showed a relationship in both race/ethnicity populations, which may add to the confidence in results. Accordingly, the associations reported in the current analysis showed consistency between non-Hispanic white and Hispanic children in stratified analyses.

Certain characteristics of the association mapping approach, namely the dependence on SNP coverage and the effect of multiple comparisons on statistical power, may have contributed to inconsistencies between results of the current study and associations reported in previous studies based on the candidate gene approach. As reviewed previously [55], the two approaches should be viewed as complementary strategies for identification of disease associated loci as they both have their respective strengths and weaknesses depending on the study being conducted. A review of the literature identified six xMHC childhood ALL candidate gene studies of non-classical HLA loci, and statistically significant associations have been reported for SNPs of the *HFE*, *HSPA1B* and *BAT3* genes [56,57,58]. None of the SNPs specifically examined in these studies were genotyped as part of the current mapping panel which precluded our ability to directly evaluate these previous associations. Indirect assessment of previously associated *BAT3* SNPs was possible through identification of proxy SNPs (r^2>0.8 in HapMap CEU) using publicly available resources [59], but evidence of an association was not observed in our study.

Any substantial effect of population stratification is likely to be minimal in the NCCLS due to the careful and detailed account of race and ethnicity obtained from the subjects and statistical adjustment. As described earlier, this is further supported by our previous report showing estimates of genetic

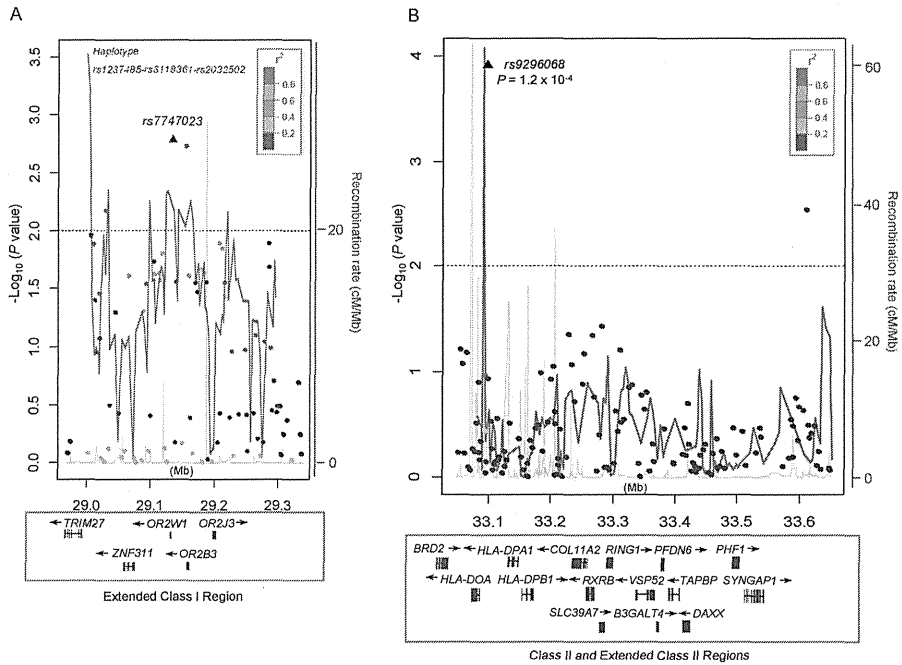

Figure 3. Plots of the two associated loci showing the results for the analysis of childhood BCP-ALL and individual SNPs (points) and three-SNP sliding window haplotypes (red lines). The -$\log_{10}$ (p-values) for each SNP (y-axis) are plotted against their chromosomal position (x-axis, Mb). The colors of the points indicate the degree of linkage disequilibrium (based on r^2) in relation to the index SNP (indicated by a black triangle). Results of the global likelihood ratio test of each three-SNP sliding window haplotype analysis are plotted and connected by the red lines. The plotted lines in blue are recombination rates (cM/Mb) based on HapMap Phase I and II data (http://hapmap.ncbi.nlm.nih.gov). A) Region A is indexed by rs7747023 as the strongest associated SNP. A statistically significant haplotype comprising rs1237485, rs3118361, and rs2032502 located adjacent to *TRIM27* was found to be associated with childhood BCP-ALL. B) Region C is indexed by a statistically significant SNP, rs9296068, located near *HLA-DOA*. A three-SNP haplotype containing rs9296068 was significantly associated with childhood BCP-ALL.

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ancestry (percent of European, Amerindian, and African ancestry) to be similar between cases and matched controls [16]. However, the effects of any potential difference in localized genetic ancestry within the MHC between cases and controls cannot be ruled out.

In this comprehensive examination of genetic variation across the xMHC, we provide evidence localizing potential disease susceptibility loci for childhood BCP-ALL to two regions, the extended class I near *TRIM27* and class II near *HLA-DOA*. Confirmation of these findings in future studies through fine-mapping and replication in other populations is warranted.

Supporting Information

Figure S1. Quantile-quantile plot of the expected versus observed -$\log_{10}$ (p-value) distribution in the analysis of 1,145 xMHC SNPs and childhood BCP-ALL risk.
Association results were derived by logistic regression assuming a log-additive genetic model and adjusting for child's age, sex, and race/ethnicity. The red line represents the plot where the observed distribution of the -$\log_{10}$ (p-value) is same as the expected distribution given the number of SNPs tested.
(TIF)

Figure S2. Linkage disequilibrium (LD) plot of the twenty SNPs associated with BCP-ALL with a p-value of less than 0.01.
The values displayed in the plot are correlation coefficients (r^2) and the intensity of shading corresponds to the *D'* measure for each marker pair. The plot and LD measures were generated separately among non-Hispanic white control children (A) and Hispanic control children (B) using Haploview (http://www.broad.mit.edu/mpg/haploview).
(TIFF)

Figure S3. Stratified analysis of childhood BCP-ALL and the potentially associated SNPs presented in Table 1 by *race/ethnicity, sex, and age group, and subgroup analyses* by major subtypes.
Odds ratios (ORs, represented by boxes with the area of each box inversely proportional to the variance of the estimate) and 95% confidence intervals (CIs, error bars) were derived using logistic regression adjusting for child's age, sex, and race/ethnicity depending on the stratification variable. The dashed vertical line represents the OR of the SNP in the analysis of BCP-ALL among all subjects and the width of the diamond is the corresponding 95% CI. $P_{homogeneity}$ was on the basis of the Cochran's Q test statistic. Abbreviations: Ca, number of case; cALL, common acute lymphoblastic leukemia; Co, number of controls.
(PDF)

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Author Contributions

Conceived and designed the experiments: KYU APC CM JLW JKW ET PB MT LFB PAB. Performed the experiments: KYU APC HH SM PR KJ AT. Analyzed the data: KYU APC SM PR. Contributed reagents/materials/analysis tools: KYU HH SM PR PT YI. Wrote the manuscript: KYU APC.

Table S1. Evaluation of the independent effects in the multivariable analysis of 20 xMHC SNPs potentially associated with childhood BCP-ALL (p-value < 0.01 in the singles SNP analysis).
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MHC and Childhood B-Cell Precursor ALL

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RESEARCH LETTER

Association Between Parental Preference and Head Computed Tomography in Children With Minor Blunt Head Trauma

Natale et al[1] recently reported that race/ethnicity is independently associated with head computed tomography (CT) use among children with minor blunt head trauma. They showed parental anxiety as an important factor influencing head CT orders in non-Hispanic white children regardless of brain injury risk.[1] In a Japanese pediatric cohort of patients with minor blunt head trauma, we conducted a study with similar objectives attempting to identify factors that influence a physician's decision to order a head CT in children.

Methods. As part of a larger hospital quality improvement activity, we conducted a cohort study comprising children younger than 15 years seen at the St Luke's International Hospital outpatient emergency clinic after experiencing a minor blunt head trauma between October 2007 and July 2012. Inclusion was limited to patients with available quality improvement data recorded by the physician onto a data collection template regarding the parents' preference for a head CT examination (deferred to physician's decision, strongly preferred, favored, and opposed) and relevant clinical data that were used to classify patients into brain injury risk categories (low, intermediate, and high) based on a validated prediction rule.[2] Electronic medical records were accessed to obtain data on whether a head CT scan was performed within 12 hours of being seen, child's age and sex, time of visit (working hours, evening, and night), and department of attending physician (pediatrics, emergency department, and other). Complete data for all variables were available for 2020 patients. This series of patients showed similar demographic characteristics and proportion of head CT examination as those patients who were not included. We used Poisson regression specifying a robust error variance and calculated relative risks (RRs) and 95% confidence intervals to evaluate factors associated with head CT order and, additionally, used a recursive partitioning method, Chi-squared Automatic Interaction Detection (CHAID),[3] to explore and visualize potential higher-order relationships that are often difficult to detect with traditional regression procedures. SPSS statistical software version 20.0 (IBM Japan Ltd) was used.

Results. Of 2020 patients, 310 (15.3%) underwent head CT scan. Using multivariate Poisson regression, head CT scan was independently associated with older age (age ≥7 years vs <2 years: RR=2.01; 95% CI, 1.57-2.57), strong parental preference (vs deferred to physician: RR=4.39; 95% CI, 3.43-5.60), high brain injury risk classification (vs low: RR=6.61; 95% CI, 4.85-9.01), and emergency department attending physician (vs pediatrics: RR=1.54; 95% CI, 1.21-1.97) (eTable, http://www.jamapeds.com). Complementary to these results, recursive partitioning based on CHAID first selected parental preference as providing the most evidence in discriminating whether a head CT scan was performed (**Figure**). Stratification also showed that nearly 40% of children in the low injury risk group underwent a head CT scan if their parents "favored" one, in contrast to only 2% of children in this risk group if the decision was deferred to the physician (Figure). Evidence suggesting higher-order interactions with child's age was observed.

Comment. The overuse of cranial CT in children,[4] even for minor blunt head trauma, is a concern particularly in light of a recent report[5] that showed CT scans in children delivering cumulative doses of about 50 mGy and 60 mGy might almost triple the risk of leukemia and brain cancer, respectively. Our results indicate that medically irrelevant factors such as parental preference may affect physician decision making and can result in unnecessary exposures to children. Furthermore, CHAID analysis suggested interaction between parental preference, injury risk classification, and child's age, but a tendency for overfitting the data is a possibility with this algorithm. Thus, additional studies would help to clarify these higher-order relationships.

Although clinical benefits likely outweigh the small risks in most cases, radiation doses from CT should be kept as low as reasonably achievable,[6] and alternative procedures should be considered, when appropriate.

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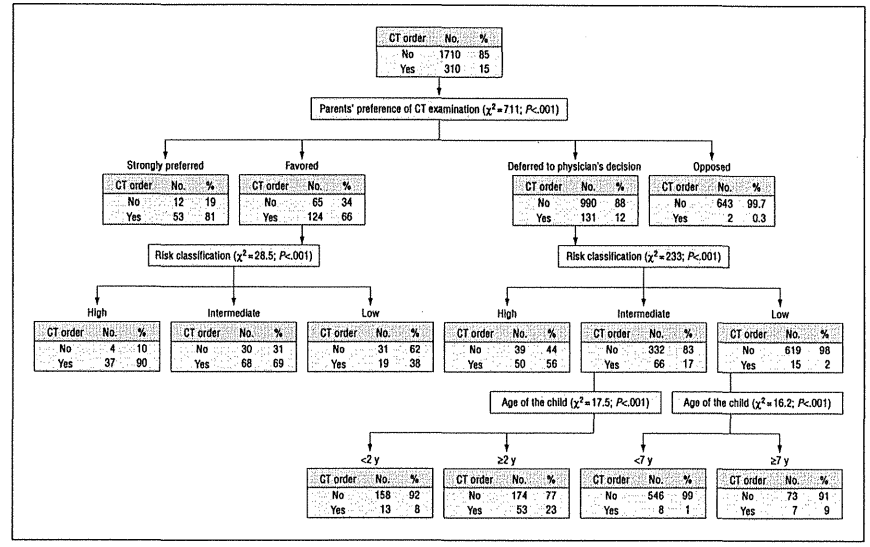

Figure. A graphical representation of the Chi-squared Automatic Interaction Detection (CHAID) analysis. Through a process of recursive partitioning based on degree of statistical significance of the χ^2 tests for independence, the CHAID algorithm evaluated which explanatory variables (eg, parental preference and brain injury risk categories), if split, most "explain" the dependent variable (head computed tomography [CT] scan). Cut points for child's age were selected by the CHAID algorithm.