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Evaluation of Polymorphisms in *EWSR1* and Risk of Ewing Sarcoma: A Report from the Childhood Cancer Survivor Study

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Abstract

Background—Ewing sarcoma is a malignant bone tumor characterized by a high frequency of somatic *EWSR1* translocations. Ewing sarcoma is less common in people of African or African-American ancestry, suggesting a genetic etiology.

Procedure—Germline DNA from white patients with Ewing sarcoma (n = 135), white controls with Wilms tumor (n = 200), and African-American controls (n = 285) was genotyped at 21 SNPs in the *EWSR1* gene. Intron 7 of *EWSR1*, the most common site of translocation, was also sequenced in all subjects. Genetic variation between groups was evaluated statistically using exact logistic regression and Fisher exact tests.

Results—One SNP in *EWSR1* (rs2857461) showed a low level of statistical association with the diagnosis of Ewing sarcoma compared to Wilms tumor. The odds ratio for having Ewing sarcoma in people with at least one copy of the minor allele of rs2857461 was 3.57 (95% confidence interval 0.79 – 21.7; p = 0.07). No other SNPs or variations in intron 7 of *EWSR1* were associated with Ewing sarcoma. The median relative difference in minor allele frequencies between white

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subjects with Ewing sarcoma and African-American controls at the evaluated *EWSR1* SNPs was 45%.

Conclusions—Variations in *EWSR1* at known SNPs or across intron 7 are not associated with the diagnosis of Ewing sarcoma. *EWSR1* does not appear to be a Ewing sarcoma susceptibility gene. The genetic basis for this disease remains unknown.

Keywords

Ewing sarcoma; *EWSR1*; single nucleotide polymorphism; genetic epidemiology

Introduction

Ewing sarcoma is the second most common type of bone cancer in children and young adults. One of the only recognized risk factors for development of this tumor is race, with a very low incidence in people of African ancestry living in both Africa and in the United States [1]. This pattern suggests a genetic component to this disease, although familial cases of Ewing sarcoma are extraordinarily uncommon [2–4]. Therefore, any genetic predisposition would be expected to have low penetrance.

Approximately 95% of Ewing sarcoma tumors harbor characteristic gene translocations involving the *EWSR1* gene [5]. These translocations result in fusion oncogenes, such as *EWSR1/FLI1* and *EWSR1/ERG* [6]. These fusion oncogenes are felt to play a critical role in the pathogenesis of these tumors and the formation of these translocations may be the precipitating event in the formation of these tumors [7,8]. When the *EWSR1* gene is translocated in Ewing sarcoma, exons 1–7 are most commonly retained in the fusion oncoprotein [9–11]. Other less common fusion oncoproteins include exons 1–9 or 1–10. Therefore, a common *EWSR1* breakpoint region has been identified as spanning from intron 7 to intron 10, with the most common breakpoints located in intron 7 [9–11]. A previous report suggested that people of African ancestry have fewer Alu repeat sequences in intron 6 of the *EWSR1* gene compared to people of European ancestry [12]. This finding suggests one possible reason for differential susceptibility to translocation, though intron 6 is not a typical site for translocation in Ewing sarcoma.

We conducted a candidate gene study to attempt to identify other genetic variations that might impact susceptibility to developing Ewing sarcoma. The primary aim was to determine if there are genetic differences in the *EWSR1* gene locus between people with Ewing sarcoma and race-matched controls without Ewing sarcoma. The secondary aim was to describe differences in the *EWSR1* gene between white and African-American populations.

Methods

Patients and Samples

The Childhood Cancer Survivor Study (CCSS) is a national cohort study of children who have survived more than 5 years from initial diagnosis between 1970 and 1986 [13]. The CCSS cohort includes 403 survivors of Ewing sarcoma. The clinical and demographic features of this group of patients have recently been published [14]. Buccal DNA has been collected from a subset of patients in the CCSS cohort, including 147 patients with Ewing sarcoma. Of these 147 patients, 135 patients self-identified as white. All 135 of these samples were included in the current study.

The CCSS tissue repository also includes buccal DNA for 548 Wilms tumor survivors. Of these, we obtained a random selection of 200 samples from self-identified white Wilms tumor survivors. This group formed the control group for our primary aim. Wilms tumor was chosen as a convenience control population since *EWSR1* is not thought to play a role in this disease and the incidence of this pediatric solid tumor does not appear to vary by race [15,16]. None of these controls had a history of Ewing sarcoma.

For DNA extraction, batched CCSS buccal cell samples were centrifuged, and cells pelleted and washed. DNA was extracted using the Puregene kit (Gentra Systems; Minneapolis, MN). DNA samples were quantified, aliquoted, and stored in hydration solution at −20 °C until used for genotyping.

We obtained control DNA samples for our secondary aim from the UCSF Genomic Resource in Arteriosclerosis (GRA). The GRA is a repository of DNA samples from more than 25,000 racially and ethnically diverse participants. Subjects in the GRA provided blood samples for isolation of germline DNA using the Puregene kit. We obtained 285 DNA samples from self-identified African-American subjects. While it is possible that some of these subjects had a history of Ewing sarcoma, the very low incidence of this disease in this population suggests otherwise.

All participants or legal guardians provided consent for secondary evaluation of their DNA samples at the time of initial specimen submission to the CCSS or to the GRA. As all samples were completely de-identified, the UCSF Committee on Human Research considered this work exempt from review.

Study Design

We evaluated variation in the *EWSR1* gene between these three groups (white Ewing sarcoma; white Wilms tumor control; and African-American control) using two approaches. First, we selected single nucleotide polymorphisms (SNPs) of interest in the *EWSR1* gene locus. The *EWSR1* locus contains 39 SNPs genotyped by the HapMap project. The Genome Variation Server of the University of Washington (http://gvs.gs.washington.edu/GVS/) was interrogated to identify the SNPs that tag these 39 SNPs with a minimum correlation coefficient (r^2) of at least 0.95, minimal genotype data coverage of 70%, and no restriction on minimal allele frequency. This process yielded an initial group of 19 tagged SNPs for genotyping in this study. Three pairs of these SNPs were in linkage disequilibrium > 95%. One of each pair was selected based on SNPs that were also selected by the Tagger program of the Broad Institute (http://www.broadinstitute.org/mpg/tagger/). For technical reasons, three of the remaining 16 SNPs could not be incorporated into the planned SNP genotyping assay. The ssSNPer SNP replacement algorithm of the Queensland Statistical Genetics Laboratory (http://gump.qimr.edu.au/general/daleN/ssSNPer/) was used to attempt to identify replacement SNPs. Replacements could be found for two of the three SNPs, yielding 15 tagged SNPs for evaluation (Supplemental Table I). In order to enrich our analysis for SNPs in the *EWSR1* breakpoint region (introns 7–10), we searched for additional validated SNPs that are not part of the HapMap project. This search yielded an additional 10 SNPs, of which 6 could be technically incorporated into our assay (Supplemental Table I).

The 21 SNPs selected for genotyping did not include any SNPs in intron 7, the most common site of *EWSR1* translocation. We therefore also sequenced the entirety of intron 7 to identify possible novel variations that might be associated with the diagnosis of Ewing sarcoma.

SNP Genotyping Techniques

Selected SNPs were genotyped using the iPLEX platform (Sequenom; San Diego; CA). Single PCR reactions were conducted with 5 μL reaction volumes of 2 mM PCR buffer, 2 mM $MgCl_2$, 500 μM of each deoxynucleotide triphosphate (dNTP), 0.1 μ M of each forward and reverse SNP specific PCR primer, 1 unit of PCR enzyme, and 10–30 ng DNA. A master mix of the above-mentioned components (minus DNA) was made for each group (plex) of SNPs and 3 μL was pipetted into each well of a 96-well plate. 2 μL of DNA was then added. PCR was then conducted on the GeneAmp PCR system 9700 (Applied Biosystems; Carlsbad, CA). Shrimp alkaline phosphatase (SAP) master mix consisting of 10x SAP Buffer and SAP enzyme was mixed and 2 μL was added to each PCR reaction. The PCR/SAP mixture was cycled at 37 °C for 40 minutes then 85 °C for 5 minutes. iPLEX extend reactions were conducted with 0.222x iPLEX Buffer, 1x iPLEX termination mix, 1x iPLEX Enzyme, and 0.74 μM – 1.75 μM of each SNP specific extend primer. 2 μL of the extend mix was added to each PCR/SAP mixture and cycled according to manufacturer specifications. 16 μL of Millipore water was added to each well. Then 6 mg of clean resin was added to each sample reaction and shaken for 20 minutes. The plate was centrifuged for 5 minutes at 3200 rpm. Sample was dispensed to a SpectroCHIP using a Nanodispenser and fired on a Sequenom MALDI-TOF spectrophotometer. Results were analyzed using MassArray Typer Version 4.0.2.0.

Sequencing Techniques

Intron 7 was sequenced using 3 target amplified regions. An additional 50–100 base pairs flanking the intron on both the 5' and 3' ends were included in the primer design to cover the intron sequence (chr22:29,683,124 – 29,684,594, hg19 University of California at Santa Cruz human genome browser build), with a 50 – 100 base pair overlap between each amplified region. The primers for the targeted regions were designed with the Primer3 algorithm (http://fokker.wi.mit.edu/primer3/input.htm). Each 2 μL PCR consisted of PCR Buffer, 2.5 mM MgCl2, 100 μM dNTP, 0.04 μL 100% DMSO, 0.2 μM of each forward and reverse PCR primer, 0.1 U of Taq polymerase and 10 ng of DNA template. PCR was then performed on the 9700 thermal cycler (Applied Biosystems). After PCR, 0.5 U of SAP and 0.5 U of exoI were added to the reactions and cycled at 37°C for 60 minutes and 95°C for 15 minutes. The sequencing reaction consisted of BigDye Terminator Mix 3.1 (Applied Biosystems), Sequencing Buffer, 200 μM of sequencing primer and 1 μL of PCR template. The sequencing reaction was performed on the 9700 thermal cycler with these conditions: 96°C for 1 minute; 25 cycles of 96°C for 10 seconds; 55°C for 5 seconds; and 60°C for 4 minutes. Xterminator (Applied Biosystems) was used after the sequencing reaction to remove impurities before testing the samples on the 3730xl DNA Analyzer (Applied Biosystems). Data were analyzed with Sequencher (Gene Codes; Ann Arbor, MI).

Statistical Methods

Genotyping data were coded based on the number of copies of the minor allele present at each position evaluated in the *EWSR1* gene. The Fisher exact test was used to evaluate statistical associations using dominant (presence of at least 1 copy of the minor allele vs. no copies of the minor allele) and recessive (presence of 2 copies of the minor allele vs. 0 or 1 copies of the minor allele) genetic models. Exact logistic regression was used to evaluate statistical associations using an additive (presence of 0 vs. 1 vs. 2 copies of the minor allele) genetic model [17]. In order to account for multiple testing, the R library [18] q-value [19] that converts p-values into corresponding false discovery rates [20] was used.

As the evaluation of variations in *EWSR1* between white and African-American populations was a planned descriptive secondary aim, no statistical comparisons were performed with

these data. Hardy-Weinberg equilibrium was tested for each SNP using the "genhwcci" module in Stata (College Station, TX), using $p < 0.05$ as the threshold criterion [21].

Results

Germline SNPs in the EWSR1 Gene in Patients with Ewing Sarcoma and Wilms Tumor

A total of 21 preselected SNPs were genotyped (Supplemental Table I). Four SNPs (rs72547438, rs72547439, rs72547473, and rs72547475) showed no variability between groups. Two additional SNPs (rs58047811 and rs72547484) showed minor allele frequencies ≤ 1.5% across the whole study population (0.8% and 1.5%, respectively). These six SNPs comprised the entire group of additional non-tagged but validated SNPs that were selected to enrich for SNPs specifically in the *EWSR1* breakpoint region. Due to low variability, these six SNPs were not evaluated further statistically. The remaining 15 SNPs were evaluated statistically for differences between white patients with Ewing sarcoma and white patients with Wilms tumor. All of these SNPs satisfied the Hardy-Weinberg equilibrium for both patient groups.

Using a dominant model, only one of the 15 evaluated SNPs showed a low level of statistical association with the diagnosis of Ewing sarcoma compared to Wilms tumor (Table I). The odds ratio for having Ewing sarcoma in people with at least one copy of the minor allele of rs2857461 (intron 1) was 3.57 (95% confidence interval 0.79 – 21.7; $p = 0.07$). After correction for multiple testing, the q-value associated with this statistical association was 0.12. The minor allele frequency at rs2857461 was 5.2% in patients with Ewing sarcoma compared to 1.5% in patients with Wilms tumor.

Recessive and additive models could be applied to data from 10 of the evaluated SNPs. Using either of these models did not reveal a statistical association between any of these 10 SNPs and the diagnosis of Ewing sarcoma.

Germline Variation in Intron 7 of the EWSR1 Gene in Ewing Sarcoma and Wilms Tumor

Sequencing of intron 7 of the *EWSR1* gene revealed little inter-subject variation. Only 9 positions in intron 7 of the *EWSR1* gene displayed any inter-subject variation in subjects with either Ewing sarcoma or Wilms tumor (Table II). Of these 9 positions, 8 positions showed variation in only a single patient. The remaining position was a previously identified SNP (rs72547435) that had not been selected *a priori* for evaluation based on our selection criteria. The minor allele frequency at this SNP did not differ between patients with Ewing sarcoma and patients with Wilms tumor.

Differences in the EWSR1 Gene between Whites and African-Americans

In order to better understand racial differences at the *EWSR1* locus, we also evaluated variations in our group of 15 SNPs across *EWSR1* in white cases with Ewing sarcoma and African-American controls (Table III). The Hardy-Weinberg equilibrium was satisfied for 12 / 15 SNPs in the African-American group.

This comparison between white cases with Ewing sarcoma and African-American controls demonstrated large differences in the minor allele frequencies in the majority of SNPs. While 3 of 15 SNPs had relative differences in minor allele frequencies of less than 20%, the remaining 12 SNPs showed relative differences in minor allele frequencies of greater than 20% between populations (median relative difference = 45%; range 0 – 154%).

We also evaluated potential genetic variation in intron 7 of *EWSR1* between white subjects with Ewing sarcoma and African-American controls. Ten positions in intron 7 of the

EWSR1 gene displayed any inter-subject variation in white subjects with Ewing sarcoma or African-American controls (Table III). Of these 10 positions, 6 showed variation in only a single patient and 1 showed variation in only 3 patients. The remaining 3 positions were previously identified SNPs that had not been selected *a priori* for evaluation based on our selection criteria. These 3 SNPs also showed large differences in the minor allele frequencies between white subjects with Ewing sarcoma and African-American controls (52 – 100% relative differences).

Discussion

This is the first candidate gene study investigating the genetic epidemiology of Ewing sarcoma. Given a possible central role for *EWSR1* somatic translocation in the pathogenesis of this disease, we focused our efforts on evaluating variation across this gene as a potential risk factor for developing Ewing sarcoma. We hypothesized that patients with Ewing sarcoma have germline genetic variants in *EWSR1* that predispose to somatic translocation at this locus. Our results argue against this hypothesis. While one *EWSR1* SNP (rs2857461) located in intron 1 was present at higher rates in patients with Ewing sarcoma compared to controls, there was only a low level of statistical evidence for this association. In addition, this SNP was present in only 5.2% of the patients with Ewing sarcoma. Therefore, even if this variation is associated with an increased risk of Ewing sarcoma, the majority of patients with Ewing sarcoma did not have this variant. Finally, this SNP is a non-coding SNP and its functional relevance is unknown.

In order to better understand the remarkably low incidence of Ewing sarcoma in people of African ancestry, we included a secondary aim to describe differences in *EWSR1* between white and African-American populations. A previous study observed differences in intron 6 of *EWSR1* between white and African populations, with an allele containing fewer Alu repeat sequences observed only in African populations [12]. In the current study, we extend this finding to show that the majority of loci evaluated across *EWSR1* demonstrate a high degree of variation between white and African-American populations. It is not clear if these variants simply reflect expected racial differences or whether these variants are associated with a lower risk of Ewing sarcoma. Future studies may focus specifically on comparing Alu repeat sequences in white patients with Ewing sarcoma and in white controls without Ewing sarcoma.

Our study is notable for a number of strengths. Given the rarity of Ewing sarcoma, we leveraged the existing resources of the CCSS to evaluate a relatively large cohort of patients with available germline DNA. We used several strategies to evaluate for possible variations in the *EWSR1* gene. By using tag SNPs, we were able to efficiently interrogate multiple regions of the gene. This point is critical since variants outside of the usual breakpoint region could nevertheless influence risk of translocation. Given that intron 7 of *EWSR1* is the most common site of translocation in Ewing sarcoma, our strategy also included full sequencing of this entire intron.

Our study is also limited by a number of weaknesses. Due to sample size considerations, we limited the number of SNPs evaluated. Our approach utilized tagged SNPs and also sequencing of intron 7. Future studies may focus on sequencing the entire *EWSR1* locus. Our SNP selection algorithm yielded only one SNP in intron 6, which had previously been shown to harbor differences based on race [12]. We attempted to incorporate additional SNPs in intron 6 into our assay, but were not able to do so due for technical reasons. In addition, the group of patients with Ewing sarcoma had all survived their disease for a minimum of 5 years in order to be included in the CCSS cohort. This point raises the possibility of a survivorship bias in which patients who survived their disease were

genetically different from patients who did not survive their disease. Therefore, the extent to which the current findings can be generalized to all patients with Ewing sarcoma remains unclear. Finally, we used a convenience sample of Wilms tumor survivors as a control group. While it is possible that these patients harbor germline *EWSR1* variants at a rate different from race-matched controls, at least two points argue against this possibility. First, the allele frequencies obtained for this group in the current study were similar to those reported in HapMap. Second, the *EWSR1* locus is not thought to be involved in the biology of Wilms tumor.

In conclusion, variations in *EWSR1* at known SNPs or across intron 7 are not associated with the diagnosis of Ewing sarcoma. Multiple loci across *EWSR1* show a high degree of variation between white and African-American groups. Additional work, including ongoing genome wide association studies in this disease, is needed to better understand the genetic basis of Ewing sarcoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table I

Odds ratio for having Ewing sarcoma (case) compared to Wilms tumor (control) in the presence of at least one copy of the minor allele of tagged SNPs in the *EWSR1* gene. P-value represents results of Fisher exact test using dominant genetic model.

SNP	Odds Ratio (95% confidence interval)	p-value
rs2301291	0.82 (0.51 – 1.30)	0.78
rs2857461	3.57 (0.79 – 21.7)	0.07
rs4820803	1.18 (0.71 – 1.97)	0.49
rs3788410	1.49 (0.79 – 2.79)	0.19
rs9613855	0.83 (0.44 – 1.56)	0.55
rs140062	1.20 (0.73 – 1.97)	0.45
rs5752899	0.83 (0.49 – 1.38)	0.44
rs11913870	1.08 (0.36 – 3.03)	0.88
rs131184	1.25 (0.74 – 2.08)	0.37
rs5763131	0.79 (0.47 – 1.32)	0.34
rs11703595	0.49 (0.08 – 2.01)	0.29
rs3761426	0.78 (0.46 – 1.32)	0.34
rs140065	1.10 (0.69 – 1.76)	0.66
rs2518683	1.44 (0.88 – 2.37)	0.13
rs174760	1.10 (0.69 – 1.76)	0.67

Table II

Variation in intron 7 of *EWSR1* in subjects with Ewing sarcoma and Wilms tumor.

Position in Intron 7	Minor Allele Frequency in Ewing Sarcoma	Minor Allele Frequency in Wilms Tumor
83	0.78% (n = 1 patient)	0%
393	0%	0.50% (n = 1 patient)
435 (rs72547435)	13.4% (n = 16 patients)	15.4% (n = 30 patients)
775	0%	0.52% (n = 1 patient)
783	0%	0.52% (n = 1 patient)
921	0.82% (n = 1 patient)	0%
924	0.82% (n = 1 patient)	0%
1051	0.79% (n = 1 patient)	0%
1193	0.79% (n = 1 patient)	0%

Table III

Variation in *EWSR1* in white subjects with Ewing sarcoma and African-American controls.

	Minor Allele Frequency in White Cases with Ewing Sarcoma	Minor Allele Frequency in African-American Controls	Relative Difference in Minor Allele Frequencies between Cases and Controls
SNP			
rs2301291	50.0%	57.2%	14.4%
rs2857461	5.2%	2.2%	57.7%
rs4820803	33.1%	30.7%	7.3%
rs3788410	34.5%	27.4%	20.6%
rs9613855	15.6%	8.3%	46.8%
rs140062	68.2%	22.6%	66.9%
rs5752899	26.9%	26.9%	0%
rs11913870	5.9%	15.0%	154.2%
rs131184	31.3%	17.3%	44.7%
rs5763131	26.9%	33.8%	25.7%
rs11703595	2.3%	0%	NA
rs3761426	25.8%	17.1%	33.7%
rs140065	43.6%	21.1%	51.6%
rs2518683	35.6%	19.4%	45.5%
rs174760	42.2%	21.4%	49.3%
Position in Intron 7			
83	0.78% (n = 1)	0%	NA
435 (rs72547435)	13.4% (n = 16)	6.4% (n = 18)	52.2%
787	0%	1.1% (n = 3)	NA
790	0%	0.36% (n = 1)	NA
907 (rs35642307)	0%	2.1% (n = 6)	100%
921	0.82% (n = 1)	0%	NA
924	0.82% (n = 1)	0%	NA
1037 (rs16987365)	0%	6.0% (n = 17)	100%
1051	0.79% (n = 1)	0%	NA
1193	0.79% (n = 1)	0%	NA

NA = Not applicable due to small numbers of cases with SNP at specific locus.