Gene 509 (2012) 263–266

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Methods Paper

Ewing's sarcoma: Analysis of single nucleotide polymorphism in the *EWS* gene

Deborah S.B.S. Silva [a], Fernanda R. Sawitzki [a], Elisa C. De Toni [a], Pietra Graebin [a], Juliane B. Picanco [a], Ana Lucia Abujamra [b,c,d,e], Caroline B. de Farias [b,c,e], Rafael Roesler [b,c,f], Algemir L. Brunetto [b,c,e], Clarice S. Alho [a,*]

[a] Laboratory of Molecular and Human Genetics, Faculty of Biosciences, PUCRS, 90619-900, Porto Alegre, RS, Brazil
[b] Cancer Research Laboratory, University Hospital Research Center (CPE-HCPA), Federal University of Rio Grande do Sul, 90035-003, Porto Alegre, RS, Brazil
[c] National Institute for Translational Medicine, 90035-003, Porto Alegre, RS, Brazil
[d] Medical Sciences Program, School of Medicine, Federal University of Rio Grande do Sul, 90035-003, Porto Alegre, RS, Brazil
[e] Children's Cancer Institute and Pediatric Oncology Unit, Federal University of Rio Grande do Sul, 90035-003, Porto Alegre, RS, Brazil
[f] Laboratory of Neuropharmacology and Neural Tumor Biology, Department of Pharmacology, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, 90050-170, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Accepted 4 August 2012
Available online 20 August 2012

Keywords:
SNP
rs2301291
rs4820803
rs4820804
Ewing's sarcoma

ABSTRACT

We aimed to investigate single nucleotide polymorphisms (SNPs) in the *EWS* gene breaking region in order to analyze Ewing's sarcoma susceptibility. The SNPs were investigated in a healthy subject population and in Ewing's sarcoma patients from Southern Brazil. Genotyping was performed by TaqMan® assay for allelic discrimination using Real-Time PCR. The analysis of incidence of SNPs or different SNP-arrangements revealed a higher presence of homozygote TT-rs4820804 in Ewing's sarcoma patients ($p = 0.02$; Chi Square Test). About 300 bp from the rs4820804 SNP lies a palindromic hexamer (5′-GCTAGC-3′) and three nucleotides (GTC), which were previously identified to be in close vicinity of the breakpoint junction in both *EWS* and *FLI1* genes. This DNA segment surrounding the rs4820804 SNP is likely to indicate a breakpoint region. If the T-rs4820804 allele predisposes a DNA fragment to breakage, homozygotes (TT-rs4820804) would have double the chance of having a chromosome break, increasing the chances for a translocation to occur. In conclusion, the TT-rs4820804 *EWS* genotype can be associated with Ewing's sarcoma and the SNP rs4820804 can be a candidate marker to understand Ewing's sarcoma susceptibility.

1. Introduction

Ewing's sarcoma (EWSR1, OMIM ID: 612219), a highly malignant and metastatic sarcoma that primarily affects bone and soft tissues (Randall et al., 2010), was first described by James Ewing in 1921. To this day it is the second most common bone tumor occurring in children, adolescents and young adults, accounting for 10–15% of all primary bone tumors (Burchill, 2003; Huvos, 1991). Clinical diagnosis is usually confirmed by imaging studies and histological analysis of bone marrow and tumor samples (Fidelia-Lambert et al., 1999). However, histological examination is limited by the lack of specific markers for Ewing's sarcoma cells. Clinically, any bone can be affected by the sarcoma, but the most common sites are the lower extremities (45%), followed by the pelvis (20%), upper extremities (13%), axial skeleton and ribs (13%), and face(2%) (Burchill, 2003; Grier, 1997).

Roughly 88% to 95% of all Ewing's sarcoma cases are caused by a translocation between the *EWS* gene (locus 22q12) and two ETS transcription factors: *FLI1* (11q24) or *ERG* (21q22) (Rerin, 2000; Zucman-Rossi et al., 1997). The most common translocation involves the *EWS* and *FLI1* genes, which occurs in approximately 85% of cases, followed by translocation between the *EWS* with *ERG* gene, which occurs in about 5–10% of cases (Delattre et al., 1992; Ozaki et al., 2002; Sorensen et al., 1993; Zucman et al., 1993). Tumor development occurs because the fusion between the *EWS* and *FLI1* genes leads to the formation of a potent and highly expressed aberrant transcription factor. The chimeric *EWS*/*FLI1* transcription factor is able to activate the expression of various oncogenes (Jedlicka, 2010; Ludwig, 2008). The *EWS* gene breakpoint is located usually between exons 1 and 7 (Burchill, 2003), a region in which a significant number of single nucleotide polymorphisms (SNPs) has been described. Bhagirath et al (1995) sequenced the fusion genes *EWS*/*FLI1* and showed that the breakpoint in *EWS* occurred in an intronic region. In this region the authors identified a palindromic hexamer 5′-GCTAGC-3′ which is

Abbreviations: °C, Degrees Celsius; dbSNP, SNP DataBase; DSB, Double-strand break; DNA, Deoxyribonucleic acid; *ERG*, Ets Related Gene; ETS, E-twenty six; *EWS*, Ewing sarcoma breakpoint region 1; *FLI1*, Friend leukemia virus integration 1; HCPA, UFRGS university hospital; HWE, Hardy–Weinberg equilibrium; kb, Kilobase; mL, Microliter; NCBI, National Center for Biotechnology Information; pb, Base pair; PCR, Polymerase Chain Reaction; q, The long arm of a chromosome; SNP, Single nucleotide polymorphism; t, Translocation; TDT, Transmission Disequilibrium Test; UFRGS, Federal University of Rio Grande do Sul.

* Corresponding author at: Laboratory of Molecular and Human Genetics, Faculty of Biosciences, PUCRS, Av. Ipiranga 6681, 90619-900, Porto Alegre, RS, Brazil. Tel./fax: +55 51 3320 3568.
E-mail address: csalho@pucrs.br (C.S. Alho).

0378-1119
http://dx.doi.org/10.1016/j.gene.2012.08.012

associated with this gene's breaking point; they also identified a common sequence of three nucleotides (GTC) at the fusion junction. However, little is still known about the *EWS* gene breaking region. We hypothesized that some sequences could promote a higher susceptibility to chromosomal breakage. Here, we investigated SNPs into the *EWS* gene breaking region in a healthy subjects' population and in Ewing's sarcoma patients from Southern Brazil.

2. Materials and methods

2.1. Subjects

Our control population consisted of at least 200 healthy DNA donors from Southern Brazil. Southern Brazil is composed by a singular genetic background: most subjects are of European origin (Portuguese, Italian, Spanish, and German ancestry) and a small amount of individuals with African traits contribute to this genetic pool (Parra et al., 2003; Pena et al., 2011). We also enrolled in this study 24 Ewing's sarcoma patients (geographically matched with the control group) and 54 of their family members, including parents and siblings. This study was approved by the institutional Ethics Committee (Protocol # 11/05424), and informed consent was obtained from all participants.

2.2. Patient phenotyping

Ewing's sarcoma patients were diagnosed at the Pediatric Oncology Unit of the UFRGS university hospital (HCPA). Of the 24 patients, 14 (58.3%) were female and 10 (41.7%) were male, with a median age of 11, ranging from 1 to 21 years old. Clinical diagnosis was confirmed by X-rays images, exploratory surgeries, or biopsies of muscle, tumor, bone marrow and/or cerebrospinal fluid obtained from lumbar puncture. The majority of the tumors were located in the lower extremity bones (45.8%) (Table 1).

2.3. Selection of SNPs

The selection of SNPs was based in three criteria: SNPs inside the *EWS* gene break point region; SNPs that had been validated and/or functionally tested; and SNPs described in the HapMap dbSNP (SNP DataBase — http://www.ncbi.nlm.nih.gov/snp) with a rare allele frequency of ≥1% in populations of European or Caucasian ethnicity. From 294 *EWS* SNPs, we pre-selected 20 and submitted to the TaqMan® SNP Genotyping Assays protocols (http://www.appliedbiosystems.com.br). After further analysis of validation and SNP localization, we selected three sequences as major candidate *EWS* SNPs: rs2301291 (intron 1, G>A), rs4820803 (intron 3, G>C) and rs4820804 (intron 3, T>C).

2.4. EWS SNP genotyping

DNA was extracted from peripheral blood leukocytes following the protocol of Lahiri and Nurnberger (1991). Genotyping was performed by TaqMan® assay for allelic discrimination using a 7500 Real-Time PCR System (Applied Biosystems, Foster, CA., USA; Life Technologies Corporation, USA) and analyzed using the allelic discrimination endpoint analysis mode of the Sequence Detection software package, SDS Version 1.3.1. We used a quality control system to ensure genotyping accuracy: sequencing verification of amplified DNA fragment, negative controls, and repetitions.

Sequencing was performed in 10% of the samples to ensure the reliability of the results obtained with the real-time PCR analysis. The primers used to amplify the targeted regions were designed using the Primer3 algorithm (http://frodo.wi.mit.edu/primer3) and are presented in Table 2. Amplification of samples was performed in a final volume of 25 μL, in a reaction containing 0.1–1 ng of DNA in 1 μL, 12.5 μL of TaqMan Universal PCR MasterMix (Applied Biosystems, Foster, CA, USA), 1 μL of 10 pmol/μL forward and reverse primers and Milli-Q water. PCR was performed on Veriti Thermal Cycler (Life Technologies Corporation, USA) using the following conditions: denaturation at 95 °C for 10 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR products were purified using ethanol precipitation. Sequencing was conducted under the BigDye™ terminator cycling conditions and performed using an Automatic Sequencer ABI 3730XL (Applied Biosystems, Foster, CA., USA; Life Technologies Corporation, USA).

2.5. Statistical analysis

Using Fisher's or Pearson's Chi-Square Test, we compared genotypic and allelic frequencies between groups, and tested the Hardy–Weinberg equilibrium (HWE). Haplotypes were assembled using Phase software v 2.1.1 (Stephens and Donnelly, 2003; Stephens et al., 2001) and Arlequin software v 3.5.x (Excoffier and Lischer, 2010). For genotype comparisons between patients and control group, we tested the three genotypes independently and combined. For an association analysis, we tested all possible haplotype constructions. Transmission Disequilibrium Test (TDT) was performed to determine if there was a presence of genetic linkage between allelic inheritance and Ewing's sarcoma. A p-value<0.05 was assumed as significant in all tests conducted.

3. Results and discussion

Ewing's sarcoma is a rare disease with a frequency of 2–3 per million in Caucasian populations in Western countries (Tortajada et al, 2005). Given this information and the fact that the state of Rio Grande do Sul has a population of 10.5 million people (IBGE Census 2010 — http://www.censo2010.ibge.gov.br/dados_divulgados/index.php?uf=43), an incidence of 21–35 affected individuals in the state is expected. We recruited 24 patients for this study. Therefore, even though our sample number may seem low, it is in accordance with the number of cases of Ewing's sarcoma expected to occur in the state of Rio Grande do Sul.

We genotyped three SNPs in introns 1 and 3 of the *EWS* gene. All SNPs were in HWE for the control group (p>0.05; Chi-Square Test). Allelic and genotypic frequencies were calculated and compared with those obtained by the HAPMAP project, and significant differences were found (Table 3). The Southern Brazilian population is known to have predominantly European inheritance as well as rarer

Table 1
Bone affected by Ewing's sarcoma in the 24 patients studied.

Affected bones	Patients [N (%)]
Lower extremities (7 femur; 1 fibula; 2 tibia)	11 (45.8)
Pelvis	4 (16.7)
Upper extremities (2 humerus)	2 (8.3)
Axial skeleton and ribs (1 cervical spine; 4 chest)	5 (20.8)
Face (2 jaw)	2 (8.3)

Table 2
Primer sequences used to amplify the targeted regions of single nucleotide polymorphisms (SNPs) designed utilizing the Primer3 algorithm (http://frodo.wi.mit.edu/primer3), expected annealing temperature, and expected amplicon size.

SNP	Primer sequence (5′–3′)	Annealing temperature	Product length
rs2301291	Forward: CGGGTGAGTATGGTGGAACT Reverse: GGGGAGGGGAGAGGAATG	60 °C	176 bp
rs4820803	Forward: TGAGTGAACCAGATAAGGGATAAC Reverse: CCATATGCCTGCTCCAAAAA	60 °C	198 bp
rs4820804	Forward: TGTTTTTGATTTTGGTTCTCCA Reverse: CCCATAGGTTGCAGTGGTCT	60 °C	179 bp

African and Asian traits (Parra et al., 2003). Our control group was important as a representative population sample, which allowed us to know the frequency of each allele in the Southern Brazilian population. The variability between the populations could be explained, at least, due to geographic particularities (especially considering the founder effect), or because of the reduced sample size investigated (<1000 subjects). The particularities of each ethnic composition (genetic background) could also be a factor to explain the differences or similarities among the populations.

To investigate if some SNP or SNP-arrangement could have different incidences in the affected individuals, we performed association studies comparing genotypic frequencies of rs2301291, rs4820803, and rs4820804 and all possible genotype combinations between Ewing's sarcoma patients and the control group. The non-combined analysis revealed a higher presence of homozygote T-rs4820804 in Ewing's sarcoma patients (Table 4). A grouped analysis of all SNPs (possible SNP-arrangements inheritance and/or haplotype combinations) was performed to analyze if a combined heritage could present a synergistic effect on the disease, but we could not detect a more important significant result (all $p > 0.05$; Chi-Square Test). Our findings corroborate that of Dubois et al. (2011), in which there was a lack of association between SNPs rs2301291 and rs4820803, and Ewing's sarcoma. The authors analyzed both SNPs in a North American population and did not find any significant results. In fact, we found that the TT-rs4820804 *EWS* genotype was the only factor significantly higher in patients with the Ewing's sarcoma phenotype.

To examine if any SNP could have been preferentially inherited by affected individuals, we performed a Transmission Disequilibrium Test comparing data from Ewing's Sarcoma patients and from their families. No statistically significant result was found (individually rs2301291: $p = 0.749$; rs4820803: $p = 0.759$; rs4820804: $p = 0.274$; Transmission Disequilibrium Test).

Table 3
Allelic and genotypic frequencies of rs2301291, rs4820803 and rs4820804 SNPs in control subjects from Southern Brazil and comparison with data from the populations investigated by the HAPMAP Project.

	Controls N (%)	HAPMAP[a]		
		European N (%)	African N (%)	Asian[b] N (%)
rs2301291	N = 206	N = 226	N = 226	N = 258
AA	17 (8.3)	34 (15)	18 (8)	106 (41)
AG	85 (41.3)	116 (51.3)	86 (38)	124 (48)
GG	104 (50.4)	76 (33.6)	122 (54)	28 (11)
P		<0.001	0.7621	<0.001
A	119 (29)	184 (40.7)	122 (27)	336 (65.1)
G	293 (71)	268 (59.3)	330 (73)	180 (34.8)
P		<0.001	0.5356	<0.001
rs4820803	N = 202	N = 226	N = 226	N = 258
CC	2 (1)	6 (2.7)	19 (8.5)	16 (6.2)
CG	64 (31.7)	70 (31)	64 (28.4)	114 (44.2)
GG	136 (67.3)	150 (66.3)	143 (63.1)	128 (49.6)
P		0.4463	<0.001	<0.001
C	68 (16.8)	82 (18.1)	90 (19.9)	146 (28.3)
G	336 (83.2)	370 (81.9)	362 (80.1)	370 (71.7)
P		0.6147	<0.001	<0.001
rs4820804	N = 208	N = 226	N = 226	N = 258
CC	34 (16.3)	18 (8)	16 (7.1)	16 (6.2)
CT	110 (52.9)	104 (46)	60 (26.5)	114 (44.2)
TT	64 (30.8)	104 (46)	150 (66.4)	128 (49.6)
P		<0.001	<0.001	<0.001
C	178 (42.8)	140 (31)	92 (20.4)	146 (28.3)
T	238 (57.2)	312 (69)	360 (79.6)	370 (71.7)
P		<0.001	<0.001	<0.001

[a] Submitter Population Handle/ID: CSHL-HAPMAP/HapMap.
[b] We considered both Asian populations HapMap-HCB (N = 86) and HapMap-JPT (N = 172). All comparisons were made between control *versus* HAPMAP population by Pearson's Chi-Square Test (for more information about HAPMAP, please visit: http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2301291, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=4820803; and http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=4820804).

Table 4
Genotype frequencies of rs2301291, rs4820803, and rs4820804 SNPs in patients with Ewing's sarcoma *versus* control group.

SNP	Genotype	Controls N (%)	Patients N (%)	p value
rs2301291	AA + AG	102 (49.5)	12 (50.0)	0.86
	GG	104 (50.5)	12 (50.0)	
rs2301291	AG + GG	189 (91.7)	20 (83.3)	0.36
	AA	17 (8.3)	4 (16.7)	
rs4820803	CC + CG	66 (32.7)	9 (37.5)	0.81
	GG	136 (67.3)	15 (62.5)	
rs4820803	CG + GG	200 (99.0)	24 (100.0)	0.56
	CC	2 (1.0)	0 (0.0)	
rs4820804	CC + CT	144 (69.2)	10 (41.7)	0.02*
	TT	64 (30.8)	14 (58.3)	
rs4820804	CT + TT	174 (83.7)	18 (75.0)	0.45
	CC	34 (16.3)	6 (25.0)	

* $p < 0.05$.

Complex diseases like Ewing's sarcoma are caused by many intrinsic (genetic) and extrinsic (environmental) factors, and each factor alone has somewhat of an effect. To search for candidate genetic markers to understand a complex disease is like looking for a needle in a haystack. Even though we have studied a single segment of the *EWS* gene, it showed a very important result in the Ewing's sarcoma scenario; the effect of the TT-rs4820804 *EWS* genotype can be small, but it was strong enough to appear as a significant factor in our population.

Ewing's sarcoma and many other cancers are caused by translocations resulting in fusion genes. However, little is known about why or where chromosomes break. Blitzblau et al (2007) mapped meiotic double-strand breaks (DSBs) in budding yeast and showed that DSBs do not appear randomly, but occur most frequently in a specific band near telomeres and at high rates around the centromere. According to Gao et al. (2005), genetic studies in yeast have revealed that the breaking hotspots contain special sites for recombination initiation, and result in a heterogeneous distribution of recombination (along the chromosome length or across the genome). In humans, more knowledge concerning the hotspots for chromosome breakage could aid in better understanding certain aspects in cancer. We believe that the presence of a particular SNP or the combination of different SNPs in a certain sequence could make a region more fragile and prone to a chromosome break. The rs4820804 SNP is a common and non-functional polymorphic variation that might be near a breakpoint region. Bhagirath et al (1995) sequenced the fusion genes *EWS/FLI1* and identified the breakpoint in *EWS* in an intronic region. The authors observed the presence of a palindromic hexamer (5′-GCTAGC-3′) and a common three-nucleotide sequence (GTC) in the close vicinity of the breakpoint junction in both *EWS* and *FLI1*. The presence of the palindromic hexamer flanking the breakpoint region was considered the most intriguing finding since palindromes may serve as specific recognition sites for DNA-binding proteins or restriction enzymes. This palindrome may have a functional significance in the genesis of the t(11;22) translocation (Bhagirath et al., 1995; Warren and Green, 1985). We analyzed the genomic context of the rs4820804 SNP and found the same palindromic hexamer (5′-GCTAGC-3′) and the same three nucleotides (GTC) about 300 bp from the SNP (Fig. 1). The DNA segment surrounding the rs4820804 SNP is likely a candidate for a breakpoint region.

Analyzing the results, the TT-rs4820804 genotype could be associated, even if not directly, with a higher propensity of *EWS* breakage or to a *EWS/FLI* translocation. In theory, one single chromosome change (heterozygosis) would be enough to lead to a translocation between the *EWS* and *ETS* genes; we call attention to the fact that we did not find a higher frequency of rs4820804 heterozygosis in patients affected

Fig. 1. Sequence context of the rs4820804 SNP (boxed) in intron 3 (GI|224589814:29663998–29696515). The palindromic hexamer 5′-GCTAGC-3′ is underlined once and the three nucleotides (GTC) are underlined twice.

by Ewing's sarcoma. If the T-rs4820804 allele could make a region fragile and predisposed to breakage, homozygotes (TT-rs4820804) would have doubled the chance to have a chromosomal breakage, increasing the chances for a translocation to occur. We can suggest the SNP rs4820804, in its genomic context, as a candidate marker to help understand the susceptibility for Ewing's sarcoma.

4. Conclusion

The present study provides statistically founded evidence that the TT-rs4820804 *EWS* genotype is associated with Ewing's sarcoma. Moreover, this polymorphism and its genomic context can provide insights into the *EWS* chromosome breakage context of tumorigenesis.

Acknowledgments

This work was supported by FAPERGS, the National Institute for Translational Medicine (INCT-TM) and the Children's Cancer Institute (ICI-RS). Deborah Soares Bispo Santos Silva, Fernanda Rosa Sawitzki, Juliane Bentes Picanço and Pietra Graebin are supported by CAPES. We would also like to thank Dr. Diógenes S. Santos, Dr. Luiz Augusto Basso and Ana Cristina de O. Dias.

Conflict of interest

The authors hereby declare that there are no conflicts of interest which may have affected the results and discussion provided herein.

References

Bhagirath, T., Abe, S., Nojima, T., Yoshida, M.C., 1995. Molecular analysis of a t(11;22) translocation junction in a Ewing's sarcoma case. Genes Chromosomes Cancer 13 (2), 126–132.

Blitzblau, H.G., Bell, G.W., Rodriguez, J., Bell, S.P., Hochwagen, A., 2007. Mapping of meiotic single-stranded DNA reveals double-stranded-break hotspots near centromeres and telomeres. Curr. Biol. 17 (23), 2003–2012.

Burchill, S.A., 2003. Ewing's sarcoma: diagnostic, prognostic, and therapeutic implications of molecular abnormalities. J. Clin. Pathol. 56, 96–102.

Delattre, O., et al., 1992. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature 359, 162–165.

DuBois, S.G., et al., 2011. Evaluation of polymorphisms in EWSR1 and risk of Ewing sarcoma: a report from the Childhood Cancer Survivor Study. Pediatr. Blood Cancer 59, 52–56.

Excoffier, L., Lischer, H.E.L., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10, 564–567.

Fidelia-Lambert, M.N., Zhuang, Z., Tsokos, M., 1999. Sensitive detection of rare Ewing's sarcoma cells in peripheral blood by reverse transcriptase polymerase chain reaction. Hum. Pathol. 30 (1), 78–80.

Gao, L., Mu, X.Q., Lin, Y., Li, Y.G., Cheng, Z.K., 2005. Meiotic recombination hotspots in eukaryotes. Yi Chuan 27 (4), 641–650.

Grier, H.E., 1997. The Ewing family of tumors. Ewing's sarcoma and primitive neuroectodermal tumors. Pediatr. Clin. North Am. 44, 991–1104.

Huvos, A.G., 1991. Ewing's sarcoma, Bone Tumors: Diagnosis, Treatment and Prognosis, Second Ed. Sanders, Philadelphia, pp. 523–552.

Jedlicka, P., 2010. Ewing sarcoma, an enigmatic malignancy of likely progenitor cell origin, driven by transcription factor oncogenic fusions. Int. J. Clin. Exp. Pathol. 3 (4), 338–347.

Lahiri, D.K., Nurnberger Jr., J.I., 1991. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic Acids Res. 19 (19), 5444.

Ludwig, J.A., 2008. Ewing sarcoma: historical perspectives, current state-of-the-art, and opportunities for targeted therapy in the future. Curr. Opin. Oncol. 20 (4), 412–418.

Ozaki, T., et al., 2002. Population-based genetic alterations in Ewing's tumors from Japanese and European Caucasian patients. Ann. Oncol. 13, 1656–1664.

Parra, F.C., Amado, R.C., Lambertucci, J.R., Rocha, J., Antunes, C.M., Pena, S.D., 2003. Color and genomic ancestry in Brazilians. Proc. Natl. Acad. Sci. U. S. A. 100, 177–182.

Pena, S.D., et al., 2011. The genomic ancestry of individuals from different geographical regions of Brazil is more uniform than expected. PLoS One 6, e17063.

Randall, R.L., et al., 2010. Is there a predisposition gene for Ewing's sarcoma? J. Oncol. 2010, 1–6.

Rerin, J.S., 2000. Analysis of Ewing Tumors by Comparative Genomic Hybridization (CGH). University of Westfäliche Wilhelms.

Sorensen, P.H., et al., 1993. Reverse transcriptase PCR amplification of *EWS*/*FLI-1* fusion transcripts as a diagnostic test for peripheral primitive neuroectodermal tumors of childhood. Diagn. Mol. Pathol. 2, 147–157.

Stephens, M., Donnelly, P., 2003. A comparison of Bayesian methods for haplotype reconstruction from population genotype data. Am. J. Hum. Genet. 73 (5), 1162–1169.

Stephens, M., Smith, N., Donnelly, P., 2001. A new statistical method for haplotype reconstruction from population data. Am. J. Hum. Genet. 68, 978–989.

Tortajada, J.F., et al., 2005. Factores de riesgo para los tumores óseos malignos pediátricos. Ann. Pediatr. 63, 537–547.

Warren, G.J., Green, R.L., 1985. Comparison of physical and genetic properties of palindromic DNA sequences. J. Bacteriol. 161, 1103–1111.

Zucman, J., et al., 1993. Combinatorial generation of variable fusion proteins in the Ewing's family of tumours. EMBO J. 12, 4481–4487.

Zucman-Rossi, J., Batzer, M.A., Stoneking, M., Delattre, O., Thomas, G., 1997. Interethnic polymorphism of EWS intron 6: genome plasticity mediated by Alu retroposition and recombination. Hum. Genet. 99, 357–363.