Research Paper

CD99 polymorphisms significantly influence the probability to develop Ewing sarcoma in earlier age and patient disease progression

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ABSTRACT

Ewing sarcoma (EWS), the second most common primary bone tumor in pediatric age, is known for its paucity of recurrent somatic abnormalities. Apart from the chimeric oncoprotein that derives from the fusion of *EWS* and *FLI* genes, recent genome-wide association studies have identified susceptibility variants near the *EGR2* gene that regulate DNA binding of *EWS-FLI*. However, to induce transformation, *EWS-FLI* requires the presence of additional molecular events, including the expression of CD99, a cell surface molecule with critical relevance for the pathogenesis of EWS. High expression of CD99 is a common and distinctive feature of EWS cells, and it has largely been used for the differential diagnosis of the disease. The present study first links *CD99* germline genetic variants to the susceptibility of EWS development and its progression. In particular, a panel of 25 single nucleotide polymorphisms has been genotyped in a case-control study. The *CD99* rs311059 T variant was found to be significantly associated [*P* value = 0.0029; OR_{het} = 3.9 (95% CI 1.5-9.8) and OR_{hom} = 5.3 (95% CI 1.2-23.7)] with EWS onset in patients less than 14 years old, while the CD99 rs312257-T was observed to be associated [*P* value = 0.0265; OR_{het} = 3.5 (95% CI 1.3-9.9)] with a reduced risk of relapse. Besides confirming the importance of *CD99*, our findings indicate that polymorphic variations in this gene may affect either development or progression of EWS, leading to further understanding of this cancer and development of better diagnostics/prognostics for children and adolescents with this devastating disease.

INTRODUCTION

Ewing's sarcoma (EWS) is a highly malignant musculoskeletal tumor that preferentially displays aggressive growth, with approximately 30% of patients harboring disseminated metastatic disease at the time of diagnosis, the most common sites being the lungs and/or bones [1]. EWS arises abruptly in pediatric age, with the peak of incidence in the second decade of life, and its natural history is still mostly unknown. The tumor is more common in Caucasians while it rarely appears in individuals of African or Asian heritage [2–6]. This observation together with reports indicating EWS in siblings or cousins [7, 8], suggests that genetic susceptibility factors may exist for this tumor, particularly among European population. However, due to the rarity of the disease, only sporadic information is available. Genome-wide scan for EWS susceptibility loci identified two common variants associated with higher susceptibility to EWS in French population [9]: a variant mapping in 1p36.22 and located proximal to the *TARDBP* gene (Tat activating regulatory DNA-binding protein, or TDP-

43, transactive response DNA-binding protein) and a variant in 10q21, located in a 561kb LD block containing three described genes: *ADO* (encoding cysteamine dioxygenase), *ZNF365* (encoding zinc-finger protein 365) and *EGR2* (encoding early growth response protein 2). Mechanistic studies connecting expression of these gene variants to EWS susceptibility have clearly demonstrated a functional association between *EGR2* and the oncogenic protein of EWS [10], the chimera *EWS-FLI*. EWS is indeed characterized by a chromosomal translocation between the *EWS* (locus 22q12) and *ETS* transcription factor genes, especially *FLI1* (11q24) (occurring in 85% of cases), or *ERG* (21q22) (about 5-10% of cases) [11, 12]. *EWS-FLI* gene fusion leads to the formation of a chimeric protein that drives the malignant phenotype by affecting both gene transcription [13–15] and RNA splicing [16, 17] of specific downstream target genes. The crucial role of *EWS-FLI* in EWS pathogenesis has been confirmed also by the genomic landscape of EWS [18–20], which showed a very low somatic mutation rate in this neoplasia. Analysis of SNPs in the *EWS* gene revealed that the rs4820804 TT genotype could be associated with a higher propensity of *EWS* breakage, increasing the chances for the translocation to occur [21]. However, in recent years it has become clear that the *EWS-FLI* oncogenic activity is a necessary but insufficient condition. To induce transformation, *EWS-FLI* requires a permissive cellular background and the presence of additional molecular events, including disruption of p53 and CD99 expression [22, 23]. CD99 is a cell surface molecule of 32 KDa [24] encoded by the pseudoautosomal *MIC2* gene, which is involved in crucial biological processes like migration, cell death, transendothelial migration of leukocytes, differentiation of T cells and thymocytes and transport of surface molecules [25–32]. CD99 is constantly present at high levels in EWS cells [33, 34] and its detection is routinely used for differential diagnosis. The EWS-FLI oncogenic activity [35] is facilitated by CD99 [23] and consistently, EWS-FLI maintains high levels of CD99 expression [23, 36, 37] either directly, through binding of *CD99* promoter [23, 37, 38] or indirectly through miRNA regulation [39]. Abrogation of CD99 in EWS cells leads to terminal neural differentiation and severely reduces tumor growth and bone metastasis in mice [23], supporting a central role for CD99 in the pathogenesis of EWS.

In the present study, we examined the genetic influence of *CD99* polymorphisms on EWS susceptibility in a representative sample of the Italian population. We were specifically interested to identify, in a case-control study, any potential genetic markers associated with age of diagnosis and to establish whether *CD99* polymorphisms may influence the EWS disease progression. Analyses revealed for the first time evidence of two variant alleles in the *CD99* gene: one (rs311059-T) strongly associated with earlier onset of EWS, and the other one (rs312257-T) related to patient event-free survival (EFS).

RESULTS

Impact of the *CD99* SNPs on the risk to develop EWS

Genotyping was carried out on a cohort of 100 EWS patients (clinicopathological features are summarized in Table 1). The genotyping iPlex assay included 25 SNPs mapping on *CD99* gene. Nine SNPs (rs311036, rs311092, rs311095, rs312199, rs313089, rs5982836, rs6567640, rs2267799, and rs311088) were excluded from statistical analyses because genotyping success rate was lower than 90%; the remaining 16 SNPs were anyhow adequate to detect association between CD99 and the risk of EWS. Deviation from Hardy-Weinberg equilibrium was observed for the SNPs rs5939307 and rs5939113 in both case- and control-group (P value < 0.001), although no apparent technical genotyping issues could be highlighted. In both circumstances the observed heterozygote frequency was lower than expected. Data from these SNPs were anyway tested for association, taking into account that caution is needed in case of deviation from the null hypothesis.

No evidence of association with alleles or genotypes was observed when EWS cases and controls were compared (Supplementary Table S1). Similarly, analysis carried out on haplotypes did not mark any association between EWS and *CD99* SNP alleles (data not shown). However, when patients were sub-grouped by age (≤14 and >14 years old), a significantly higher frequency of the *CD99* rs311059-T variant was observed in those showing an earlier onset of the disease (P value = 0.0029) [OR_{het} = 3.9 (95% CI 1.5-9.8) and OR_{hom} = 5.3 (95% CI 1.2-23.7)] (Table 2). The association test was significant at the Bonferroni corrected threshold level for multiple testing ($\alpha < 0.0031$). A similar trend was corroborated when the EWS patients with earlier onset of the disease were compared to the control group (Table 2).

In order to improve the coverage of exon 2 region, which contains the rs311059, four additional SNPs close to this polymorphism were selected and genotyped. No evidence of association was observed between these additional markers and the predisposition to EWS. However, haplotype analysis confirmed that the haplotype rs311059-T/rs311060-C showed the same level of association with the onset of EWS before 14 years of age [P = 0.0029; OR = 2.5 (95% CI 1.2-23.7)] that was previously found for the allele rs311059-T. Moreover, to verify that *CD99* polymorphisms specifically influence the EWS onset in pediatric age, the genotyping was extended to patients with OS (clinicopathological features are summarized in Supplementary Table S2), which also frequently affects children and adolescents. No correlation was found between *CD99* rs311059-T and the risk to develop OS (data not shown), further confirming that the association between this variant and higher probability of premature disease onset is a hallmark of EWS.

Table 1: Clinicopathological features of 100 EWS patients

Characteristics	N.	%
Gender		
Female	37	37
Male	63	63
Age (3-45 years)		
≤ 14 years	35	35
> 14 years	65	65
Location		
Extremities	64	64
Pelvis	13	13
Central	23	23
Metastasis at diagnosis		
Yes	20	20
No	80	80
Local treatment		
Surgery	56	56
Surgery + RXT	22	22
RXT	21	21
Not Done	1	1
Chemoprotocol		
EWS-REN	17	17
ISG/SSG III	64	64
ISG/SSG IV	9	9
Other	10	10

Impact of the *CD99* SNPs on EWS disease progression and patient outcome

In the same cohort of EWS patients, we tested if *CD99* polymorphisms could influence disease progression. The value of *CD99* SNPs as prognostic biomarkers was calculated only in patients with localized EWS at diagnosis. Patients with metastatic disease at diagnosis were excluded from this analysis due to differences in treatment and the well-recognized negative impact of the presence of metastasis on patient outcome. The association analysis carried out on 78 patients with a minimum follow-up of 10 months (Table 3) showed that the variant rs312257-T is associated with a lower risk of EWS relapse [$P = 0.0265$; $OR_{het} = 3.5$ (95% CI 1.3-9.9)]. Although this link was not sufficiently strong to be confirmed after Bonferroni correction for multiple testing, the prognostic value of the *CD99* rs312257-T was proved to be associated with EFS of EWS patients when Kaplan-Meier survival curves were designed (Figure 1) ($P = 0.03$, log-rank test). Presence of rs312257 T variant allele as well as good response of tumors to neoadjuvant chemotherapy, which resulted significantly associated with clinical outcome in univariate analysis (Table 3), were confirmed as independent risk factors associated with good outcome by multivariate Cox's proportional hazards regression analysis (Table 4).

DISCUSSION

Although cancer is a complex disease resulting from the interplay of genetic, epigenetic, and environmental factors, the etiopathology of pediatric tumors supports the role of genetic and epigenetic alterations rather than environmental factors in the tumorigenesis. For EWS, race is one of the very few, well-recognized risk factor [40] and this supports the hypothesis of a genetic contribution to its etiology. However, familial cases of EWS are

Table 2: Association analysis between *CD99* polymorphisms and EWS age onset

SNP information		Genotype age ≤14			Genotype age >14			MAF		AA	Odds Ratio (95% CI)	
SNP ID	**Alleles***	**11**	**12**	**22**	**11**	**12**	**22**	**case ≤14**	**case >14**	***P value***	**OR_{het}**	**OR_{hom}**
rs311057	G/A	15	16	0	41	19	1	0.26	0.17	0.1692	2.30 (0.95-5.61)	0.89 (0.03-23.09)
rs311059	C/T	9	21	5	38	23	4	0.44	0.24	**0.0029**	**3.86 (1.51-9.84)**	**5.28 (1.18-23.71)**
rs311060	C/G	24	11	0	39	24	2	0.16	0.22	0.3214	0.75 (0.31-1.79)	0.32 (0.02-7.00)
rs1136447	C/T	10	17	8	11	37	17	0.47	0.45	0.3130	0.51 (0.18-1.42)	0.52 (0.16-1.72)
rs6567640	C/G	12	18	5	16	40	9	0.40	0.45	0.5294	0.60 (0.24-1.52)	0.74 (0.20-2.79)
rs311074	A/G	11	12	12	23	30	12	0.46	0.42	0.1799	0.84 (0.31-2.23)	2.09 (0.71-6.13)

SNP information		Genotype age ≤14			Genotype control			MAF		AA	Odds Ratio (95% CI)	
SNP ID	**Alleles**	**11**	**12**	**22**	**11**	**12**	**22**	**case ≤14**	**control**	***P value***	**OR_{het}**	**OR_{hom}**
rs311057	G/A	15	16	0	95	33	9	0.26	0.19	0.2005	3.07 (1.37-6.89)	0.32 (0.02-5.86)
rs311059	C/T	9	21	5	81	49	17	0.44	0.28	**0.0093**	**3.86 (1.64-9.09)**	2.65 (0.79-8.89)
rs311060	C/G	24	11	0	83	59	5	0.16	0.23	0.1591	0.65 (0.29-1.42)	0.31 (0.02-5.80)
rs1136447	C/T	10	17	8	38	70	37	0.47	0.50	0.7059	0.92 (0.39-2.21)	0.82 (0.29-2.31)
rs6567640	C/G	12	18	5	40	81	26	0.40	0.45	0.4278	0.74 (0.33-1.69)	0.64 (0.20-2.03)
rs311074	A/G	11	12	12	46	78	23	0.46	0.42	0.1610	0.64 (0.26-1.58)	2.18 (0.84-5.69)

SNP information		Genotype age >14			Genotype control			MAF		AA	Odds Ratio (95% CI)	
SNP ID	**Alleles**	**11**	**12**	**22**	**11**	**12**	**22**	**case >14**	**control**	***P value***	**OR_{het}**	**OR_{hom}**
rs311057	G/A	41	19	1	95	33	9	0.17	0.19	0.7387	1.33 (0.68-2.62)	0.26 (0.03-2.10)
rs311059	C/T	38	23	4	81	49	17	0.24	0.28	0.3477	1.00 (0.53-1.87)	0.50 (0.16-1.60)
rs311060	C/G	39	24	2	83	59	5	0.22	0.23	0.6625	0.87 (0.47-1.59)	0.85 (0.16-4.58)
rs1136447	C/T	11	37	17	38	70	37	0.45	0.50	0.3471	1.83 (0.84-3.99)	1.59 (0.66-3.84)
rs6567640	C/G	16	40	9	40	81	26	0.45	0.45	0.9054	1.24 (0.62-2.47)	0.87 (0.33-2.25)
rs311074	A/G	23	30	12	46	78	23	0.42	0.42	0.9022	0.77 (0.40-1.48)	1.04 (0.44-2.46)

Abbreviations: SNP, single nucleotide polymorphism; MAF, minor allele frequency; AA, allelic association ;OR_{het}, odds ratio for heterozygote; OR_{hom}, odds ratio for homozygote.
*Major allele is provided first.

extraordinarily uncommon [7, 8, 41] and, therefore, any genetic predisposition would be expected to have low penetrance. Considering that approximately 95% of EWS patients harbor characteristic gene translocations involving the *EWS* gene, germline *EWS* SNPs have been studied by different groups without reporting genetic variations that significantly impact on susceptibility to develop EWS [21, 42]. Using a combination of high-throughput sequencing and integrative genomics, susceptibility variants near *EGR2* gene have been identified [9]. More recently, Grünewald *et al*. [10] has demonstrated that *EGR2* significantly influences EWS growth in mice and recognized an inherited variation on chromosome 10 that affects EWS susceptibility by facilitating the binding of the EWS-FLI oncoprotein to the *EGR2* locus. Besides *EWS-FLI*, the other major common determinant of EWS is CD99 [23, 43, 44], which contributes substantially to EWS-FLI transformation.

In this paper, we have evaluated *CD99* single nucleotide variations as potential risk factors for the development of EWS. We hypothesized that EWS occurring in pediatric age (i.e. ≤14 years) could be supported by a different genetic predisposition with respect to EWS occurring later in life. Indeed, we found

Table 3: Clinicopathological features of 78 localized EWS patients

Characteristics	N.	%	Association with prognosis EFS (*P* value)
Gender			
Female	31	39.7	0.760
Male	47	60.3	
Age			
≤ 14 years	27	34.6	0.160
> 14 years	51	65.4	
Location			
Extremities	53	67.9	
Pelvis	18	23.1	0.637
Central	7	9.0	
Local treatment			
Surgery	51	65.4	
Surgery + RXT	17	21.8	0.250
RXT	10	12.8	
Chemoprotocol			
EWS-REN	16	20.5	0.540
ISG/SSG III	58	74.4	
Other	4	5.1	
Response to chemotherapy			
Good	25	32.0	
Poor	40	51.3	0.001
ND	13	16.7	
EFS (Status)			
NED	39	50.0	
REL	39	50.0	

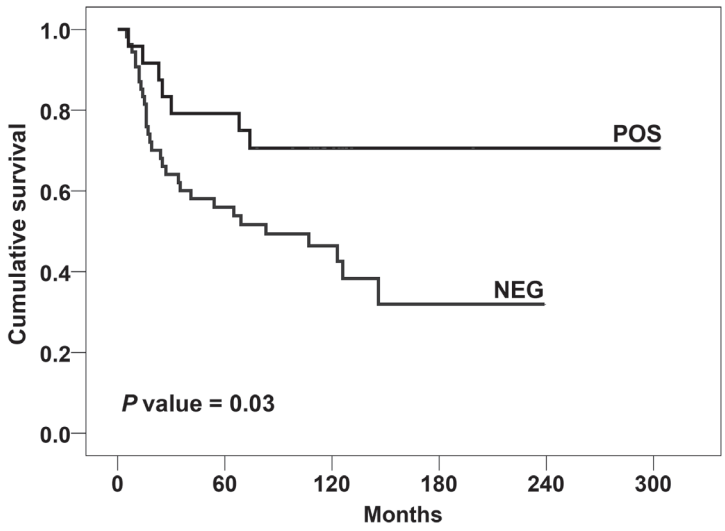

Figure 1: Prognostic impact of the presence of *CD99* rs312257 T allele according to Kaplan–Meier curves and log-rank test. EWS patients were classified for the presence (POS) or absence (NEG) of the variant. Event-free survival (EFS) was considered.

Table 4: Multivariate analysis using Cox's proportional hazards regression analysis

Variables associated with better prognosis	Adjusted risk-rate ratio EFS	CI (95%)	*P* value
Response to chemotherapy (GOOD)	0.205	(0.078 – 0.539)	0.001
Presence of T allele at rs312257 SNP	0.657	(0.429 – 1.005)	0.053

Adjusted risk-rate (RR) ratio of relapse was estimated for the variables that resulted to be significantly associated with prognosis by univariated analysis.

that the *CD99* rs311059-T is significantly associated with an increased risk to develop the disease before 14 years of age. Rs311059 is a non-coding SNP and its functional relevance is unknown. As for the inherited variation on chromosome 10 near *EGR2* [10], *CD99* rs311059 variant allele may not affect the gene product sequence, but indirectly increasing susceptibility to EWS through epigenetic regulation of gene expression. Rs311059 maps inside the suppressor of zeste 12 (*SUZ12*) binding site, as shown on UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly. *SUZ12*, a gene identified at the breakpoints of a recurrent chromosomal translocation reported in endometrial stromal sarcoma [45], encodes for a zinc finger protein belonging to the Polycomb Repressive Complex 2 (PRC2), which modulates chromatin structure by repressive mechanisms [46, 47]. PRC2 specifically stimulates H3K27 trimethylation, which is associated with transcriptional repression, and can recruit DNMTs [48], thus acting as a platform for aberrant *de novo* promoter methylation. We speculate that the presence of the rs311059 T allele creates a mismatch in SUZ12 binding site, which might facilitate the expression of *CD99*, enabling the permissive condition for EWS-FLI transformation. This could explain why rs311059-T appears to be a susceptibility factor in the EWS onset in pediatric age. The significant level of rs311059-T presence in young patients was found to be a distinctive feature of this disease. In fact, in patients affected by OS, which shares with EWS both the skeletal localization and the peak onset in childhood and adolescence age, the genotyping test did not provide any evidence of association.

In EWS, CD99 is expressed at high levels in virtually all samples [34, 49] but no correlation between CD99 expression and patient outcome has been highlighted so far. In this paper, we first identified the *CD99* rs312257 T genetic variant as significantly associated with EFS. The presence of the T allele confers superior survival to patients with localized EWS. Although mechanistic studies are needed to explain this observation, our findings support the hypothesis that variations in *CD99* gene may significantly affect the progression of EWS. Considering the rarity of the tumor, we offer this evidence to the scientific community for more extensive validation studies.

MATERIALS AND METHODS

Clinical samples

A cohort of 100 unrelated Italian patients with confirmed diagnosis of localized (80 cases) or disseminated (20 cases) EWS treated at the Rizzoli Orthopaedic Institute (Bologna, Italy) was considered. All cases included showed a specific *EWS-ETS* fusion, EWS patients underwent local treatments (surgery; surgery plus radiotherapy; radiotherapy only, when the surgeon considered the lesion inoperable or due to patient refusal) and neo-adjuvant chemotherapy according to protocols that were previously reported in detail [50, 51]. Clinicopathological data are shown in Table 1.

Patients with localized EWS were followed-up and clinical information updated (median follow-up: 76 months, range: 5-304 months). EFS was calculated from the date of initial diagnosis to clinical endpoint, which is considered as the time of occurrence of adverse events (defined as recurrence or metastases at any site for EFS). Histological response to chemotherapy was evaluated according to the method proposed by Picci *et al*. [52].

The control group consisted of 147 unrelated healthy volunteers with matching sex, ethnic origin and from the same geographical area (Italy). In addition, a cohort of 121 osteosarcoma (OS) samples was also enrolled for this study as a further comparison group. OS was chosen because it shares with EWS the same site of origin (bone) and a similar peak of incidence in juvenile age, despite being a very different type of tumor either from biological and genetic point of view [53]. Clinicopathological data of OS samples are shown in Supplementary Table S2. The ethical committee of the Rizzoli Institute approved the studies and informed consent was obtained.

Single nucleotide polymorphism genotyping

DNA was extracted from peripheral blood leukocytes using standard DNAzol procedure (Thermo

Fisher Scientific, Foster City, CA, USA). DNA quality and concentration were evaluated by Nanodrop (Thermo Fisher Scientific). Aliquots of 20 µl at the concentration of 12 ng/µl for each sample were employed for genotyping by the Sequenom MALDI-TOF mass spectrometer MassArray system (as a service at Applied Biomedical Research Center, S. Orsola-Malpighi Polyclinic, Bologna, Italy). To perform genotyping, the genotypic data of Caucasian and Italian population, collected by the International HapMap Consortium (CEU+TSI dataset), were evaluated by the HaploTagger software to explore the haplotype complexity of *CD99* locus. We imposed that tag SNPs had to capture at least 80% of the *CD99* alleles having a minor allele frequency > 0.1. We selected, therefore, 25 tag SNPs that best represent the genomic structure of *CD99* with the minimal redundancy level [54]. Supplementary Figure S1 shows the position of each of the 25 SNPs along the *CD99* locus and haploblock structures [54]. Assay design and analysis of allele peaks were performed using specific Sequenom software (Sequenom, San Diego, California, USA). Primers were synthesized at Metabion (Martinsried, Germany). In order to improve the coverage of the gene, the genotyping of 4 additional SNPs selected by Haplotagger was carried out by using a high resolution melting curve (HRM) analysis approach. Ten ng of DNA from each sample was amplified by using the MeltDoctor™ HRM Master Mix and the Applied Biosystems ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific), according to supplier's suggestions. The sequence of control samples was assessed by Sanger Sequencing and used as reference melt curve profile for the different genotypes.

Statistical analysis

The deviations from Hardy-Weinberg equilibrium for genotype distributions, in both patient and control groups, were examined using Pearson's χ^2 test.

Analysis for genotypic and haplotypic associations were performed using UNPHASED program (Version 3.1.7) which employs an allelic likelihood ratio test [55]. Odd ratios were calculated in order to estimate the level of association of the rare allele carriers, i.e. heterozygotes *vs* non-carriers, as well as homozygotes *vs* non carriers. The results were adjusted for multiple testing with the Bonferroni correction according to the number of tag SNPs analyzed. Bonferroni correction is considered an overly stringent adjustment when tests are not independent, such as in case of linkage disequilibrium between SNPs.

Patients were then sub-grouped by age at diagnosis into 2 groups (≤14 and >14 years old) and association analysis was performed. Comparisons as follows: 1) ≤14 *vs* >14 years old; 2) ≤14 years old *vs* control group; 3) >14 years old *vs* control group. The association was also tested for 4 additional SNPs (rs1136447, rs311060, rs6567640, and rs311074) selected by Haplotagger, covering the exon 2 and the boundaries. These four SNPs together with rs311057 and rs311059, mapping near to the 5' exon 2 boundary were considered for haplotype analysis (until six marker combinations).

To verify association between *CD99* SNPs and patient prognosis, a subgroup of 78 patients with localized EWS having a minimum follow-up of 10 months was considered. For this subgroup, Kaplan-Meier and log-rank tests were used to draw and evaluate the significance of survival curves in EWS patients in relation to *CD99* SNP alleles.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed by the authors.

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