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RESEARCH

Expression of the Chemokine Receptors CXCR3, CXCR4, CXCR7 and Their Ligands in Rhabdomyosarcoma

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Abstract Rhabdomyosarcomas (RMS) are soft tissue malignant tumors of childhood and adolescents. The mechanisms underlying their aggressiveness are still poorly understood. Chemokines are chemotactic proteins involved in pathological processes that have been intensely studied in several types of cancers because of their influence in migration, angiogenesis, or metastases. We analyzed the expression of the chemokine receptors CXCR3, CXCR4 and CXCR7 and their ligands CXCL9, CXCL10, CXCL11 and CXCL12, in 15 RMS samples derived from nine patients. Expression was measured in tumors and primary cultures of RMS by Real-Time Polymerase Chain Reaction, immunostaining and flow cytometry. Our results show that these receptors are widely expressed in RMS. A significant difference between CXCL12/CXCR4, CXCL12/CXCR7, CXCL11/CXCR7 expression ratios was found in alveolar versus embryonal RMS and similarly between CXCL12/CXCR4 and CXCL11/CXCR3 ratios in primary versus recurrent tumors. These findings suggest a possible association between the interrelation of chemokine/chemokine-receptor and an aggressive biological behavior in RMS.

Keywords Alveolar Rhabdomyosarcoma · Chemokines · Embryonal Rhabdomyosarcoma · Flow cytometry

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Introduction

Rhabdomyosarcoma (RMS) is the most common soft-tissue neoplasm of childhood and adolescence. As recurrence and metastasis are quite frequent, it is pivotal to study the underlying mechanisms involved in these processes in order to prevent therapeutically the metastatic events [1]. Histologically, there are two main subgroups, alveolar RMS (ARMS) and embryonal RMS (ERMS) which present completely separate genetic abnormalities. ARMS is more aggressive and frequently have a bad outcome; cytogenetics show a characteristic t(2;13)(q35;q14) translocation or its variant t(1;13)(p36;q14) in 80 % of cases [2]; these translocations result in fusion genes that encode proteins with increased transcriptional activity [3, 4]. ERMS usually shows gain of whole chromosomes but no characteristic structural anomalies [5–7].

Chemokines are chemo-attractant proteins that bind to G-coupled receptors and take part in many biological processes, such as cell adhesion and directional migration, and have been related to tumor initiation and progression. Furthermore, chemokines and their receptors have a role in cell survival and metastatic dissemination in several types of cancer, as ovarian, breast and prostate cancers, or melanoma [8–10]. Their study is difficult because different chemokines can bind the same receptor, and one chemokine can bind several receptors also, creating a wide variety of interactions that result in multiple biological outcomes.

There are numerous reports about the importance of the chemokine CXCL12 and its CXCR4 receptor in the

relationship between cancer cells and their environment in several malignancies [11–16]. CXCR7 is another receptor of CXCL12 which shows even higher affinity to CXCL12 than CXCR4, and seems to play a role in survival, cell adhesion and metastases [17, 18]. A decrease in tumor cell proliferation and migration has been described when CXCR4 is inhibited in different models of cancer [11, 19, 20]. CXCR7 is also a receptor for the chemokine CXCL11 [17], like CXCR3 [21]. CXCL11 is able to activate pathways that participate in tumor growth, but the meaning of this activation through CXCR7 is still unclear [17, 18, 21]. CXCR3 binds also CXCL9 and CXCL10 in addition to CXCL11 [22]; mice models treated with CXCR3 ligands have shown impaired metastases and inhibition of tumor growth and angiogenesis [23]. The research studies concerning these chemokines highlights the importance of studying CXCL9, CXCL10, CXCL11 and CXCL12 simultaneously to their shared receptors CXCR3, CXCR4 and CXCR7, in cancer.

There are few studies treating the expression of chemokine receptors in RMS, and even less on their chemokine ligands [24–30]. At our understanding, there are not previous studies about the expression of the above mentioned chemokines and their receptors at the mRNA level in RMS fresh samples. Herein we report the mRNA expression of all these shared chemokines and chemokine receptors in a series of human RMS. We also study the histological patterns of expression of these molecules by immunostaining and the surface expression of the receptors by flow cytometry.

Material and Methods

Tumor Samples

We analyzed 15 RMS samples derived from nine patients treated surgically at the University Clinic Hospital of Valencia (UCHV). From them, 11 were primary tumors and four were recurrences derived from them. Eight samples (4 ARMS and 4 ERMS) were inoculated into right flank of nude mice and xenografts were generated. Fresh tumor specimens were divided for histological examination, molecular studies and cell culture. The study was performed with the approval from the Institutional Review Board of the University of Valencia. Cases, source, location and additional information are summarized in Table 1; the cytogenetic features of the cases have been previously reported [6, 7].

Histopathology and Immunohistochemistry

For histopathologic examination, tumor samples were fixed in neutral buffered formalin, sectioned, and stained with hematoxylin and eosin. Immunohistochemistry was carried out for muscle-specific actin, myosin, myoglobin, desmin, and vimentin in order to confirm the diagnosis. Mouse monoclonal antibodies (mAB) against human CXCR3 (5 μg/ml); CXCR4 (5 μg/ml); CXCR7, (15 μg/ml); CXCL9, (5 μg/ml); CXCL12, (8 μg/ml); all from R&D Systems, (Abingdon, UK); mouse mAb against human CXCL10, (5 μg/ml), (BD Biosciences, Franklin Lakes, NJ, USA); and rabbit anti-human CXCL11, (5 μg/ml), (PeproTech, Rocky Hill, NJ, USA) were analyzed. Avidin-biotin peroxidase method was used and slides were counterstained with hematoxylin. Sections of well-characterized melanomas were used as positive controls for CXCR4; sections incubated with isotype-matched control immunoglobulins were used as negative controls.

For immunocytochemistry, cells were grown in Lab-Tek chamber slides (Miles Laboratories, Naperville, IL, USA). After washing with PBS, the cells were fixed with cold methanol-acetone for 5 min. The mAB used were the above mentioned: mouse mAB against human CXCR3, CXCR4, CXCR7, CXCL9 and CXCL12 (R&D Systems); mouse mAb against human CXCL10 (BD Biosciences) and rabbit anti-human CXCL11 pAb (Peprotech). The cells reacted with each of these primary antibodies for 1 h at room temperature. The attached antibodies were visualized by the avidin-biotin-peroxidase procedure (Dako, Carpentaria, CA, USA). The cell lines mel-RC-08 and Mel-Ho were used as positive controls [31, 32]. Negative controls were used in each immunoreaction.

Short-Term Cultures

To obtain isolated cells from tumors, a fragment of each biopsy was disaggregated with 0.2 % collagenase II (Sigma-Aldrich, St. Louis, MO, USA). The cells were seeded in 25-cm^2 flasks with RPMI-1640 medium supplemented with 20 % fetal bovine serum and Pen-Strep 1 % (all from Gibco, Life Technologies, Paisley, UK). The cells were grown in a humidified atmosphere with 5 % CO_2. After subculturing, cells were seeded in Lab-TekTM Chamber Slide System (Thermo-Scientific) for immuncytochemistry assays and a suspension of live cells was used for cytometric analysis.

Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from tumor frozen samples with TRIzol® and PureLinkTM Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA). RNA of each sample was assessed with NanoDROP 2000 (Thermo-Scientific) and the quality was evaluated in a 1.5 % agarose gel. Two micrograms of RNAs were reverse-transcribed to complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed using an ABI Prism 7900 HT Fast Real Time PCR System (Applied Biosystems). Target and reference genes were amplified in separate wells, and every gene assayed was run in triplicate

Table 1 Clinical data

Case	Sex/age	Subtype	Location	Treatment	Follow-up status/ Time months	Sample number	Source
1	M/5 m	ERMS	Soft tissue	S, CH	DOD/57	1	PT
						2	R
2	M/4y	ERMS	Paratesticular	S, CH	NED/70	3	PT
						4	R
						5	XPT
						6	XR
3	F/15 m	ERMS	Right tight	S, CH, RT	DOD/69	7	PT
						8	R
4	M/8y	ERMS	Orbital	S, CH, RT	NED/10	9	XPT
						10	XPT
5	M/6y	ERMS	Parotid gland	S, CH, RT	NED/53	11	XPT
6	F/18y	ARMS	Buttock	S, CH, RT	DOD/10	12	XPT
7	M/13y	ARMS	Right forearm	S, CH, RT	NED/159	13	XPT
8	F/6y	ARMS	Right calf	S, CH, RT	DOD/48	14	XPT
9	F/12y	ARMS	Periorbital	S, CH, RT	DOD/30	15	XPT

ARMS alveolar rhabdomyosarcoma, *CH* chemotherapy, *DOD* died of disease, *ERMS* embryonal rhabdomyosarcoma, *F* female, *M* male, *m* months, *NED* no evidence of disease, *PT* primary tumor, *RT* radiation therapy, *S* surgery, *XPT* xenograft from primary tumor, *XR* xenograft from recurrence, *y* years

and in the same plate. TaqMan Universal® PCR Master mix and Assays-on-demand gene expression products (Applied Byosistems) were used. qPCRs were performed following the manufacturer's instructions. Briefly: 2 min at 50^0C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C. Reference genes were *GAPDH* and *18S*. Primers used were: *CXCR3* Hs_00171041, *CXCR4* Hs_00607978, *CXCR7* Hs_00664172, *CXCL9* Hs_00970538, *CXCL10* Hs_0171042, *CXCL11* Hs_0171138, *CXCL12* Hs_00171022, *18S rRNA* Hs_99999901 and *GAPDH* Hs_99999905. qPCR data were collected using SDS 2.1 software and results were automatically analyzed by RQ Manager 1.2 software (Applied Biosystems). For the relative quantification of gene expression, the comparative Ct method was used. The final amount of the target gene, normalized to an endogenous reference gene (ΔCt=Ct target gene - Ct reference gene), was given by the formula: 2-ΔCt [33], which allowed for the comparison of the different samples of our study. The formula $2^{-[\Delta Ct \text{ chemokine target gene} - \Delta Ct \text{ chemokine receptor target gene}]}$ based in a previous work in melanoma [31], was used for the calculation of chemokine/chemokine-receptor mRNA ratios.

Flow Cytometry

For the quantification of cell surface expression of chemokine receptors, cells at subconfluency (50–70 %) were detached with 2 mM EDTA in PBS, washed and resuspended in ice-cold culture medium at $1x10^6$cells/ml. Subsequently, 100 μl of this cell suspension were incubated on ice for 30 min with the chemokine receptors mAbs and correspondent isotypic controls. All mAbs against chemokine receptors were conjugated to phycoerytrin (PE). mAbs against CXCR3, CXCR4, and correspondent isotypic controls were purchased from BD Biosciences. mAb against CXCR7 and the correspondent isotypic control were purchased from R&D Systems. After incubation, cells were washed with ice-cold PBS and resuspended in 500 μl of culture medium for flow cytometric analysis. To determine dead cells 3 μg/ml of the DNA intercalating fluorochrome 7-Aminoactinomycin D (7-AAD, Sigma-Aldrich) were added prior to cytometric analysis.

For the intracellular quantification of chemokine expression, cells at subconfluency (50–70 %) were detached with 2 mM EDTA in PBS, washed, fixed with 4 % paraformaldehyde and permeabilized with 0.1 % Triton/PBS adjusting cell suspension to 1×10^6 cells/ml. Subsequently, 100 μl volumes of this cell suspension were incubated on ice for 30 min with the chemokine unconjugated mAbs. Afterwards, cells were washed twice with ice-cold 1 % BSA/PBS and incubated on ice for 30 min with the secondary FITC-conjugated antibody. All chemokine ligands (CXCL9, CXL10, CXCL11, and CXCL12) were detected using primary mouse-anti-human mAbs from R&D Systems with a secondary goat anti-mouse antibody labeled with FITC (R&D Systems). An aliquot of 100 μl of cell suspension incubated only with the secondary FITC-conjugated antibody was used as a negative control for all the chemokine ligands. Finally, cells were washed twice and resuspended in 500 μl of ice-cold 1 % BSA/PBS for flow cytometric analysis.

Five thousand cells were analyzed per condition in triplicate, using a Cytomics FC500 MCL flow cytometer

(Beckman-Coulter, CA, US) with an argon-ion laser, and the means obtained were statistically evaluated.

Flow-Check Fluorospheres (Beckman-Coulter, CA, US) were used according to manufacturer instructions to verify instrument optical alignment and fluidics before each measurement session.

Statistical Analysis

Student's *T*-test or one-way analysis of variances using non-parametric Kruskal-Wallis test were used. Values with $p \leq 0.05$ were considered statistically significant.

Results

Immunohistological Results

The clinical data of the patients are shown in Table 1. The ARMS were composed predominantly of sheets of polygonal cells separated by fibrous septa into nests with an alveolar growth pattern. The ERMS presented small round tumor cells with hyperchromatic nuclei and abundant eosinophilic cytoplasms. All tumors showed reactivity for muscle-specific actin, desmin, myosin, and vimentin.

Immunoexpression for the chemokines receptors CXCR3, CXCR4 and CXCR7 and their ligands was found in both primary (Fig. 1 and a–j) and recurrent tumors, therefore it was possible to determine the patterns of expression: tumoral samples showed a surface expression as well as a granular cytoplasmic expression for all chemokine receptors analyzed; CXCR4 and CXCR7 were also expressed in perinuclear site (Fig. 1b and c). Furthermore, some cells (approximately 10 %) showed nuclear expression of CXCR4 (Fig. 1b). RMS-xenografts were also positive for the chemokines CXCL11 and CXCL12 (Fig. 1e); and also for chemokine receptors CXCR4 (Fig. 1d) and CXCR7 (Fig. 1f). Cells from cases 1 to 4 were well adapted to in vitro conditions (Fig. 1 and e–h). Cultured cells also showed expression of all chemokine receptors showing a granular pattern of CXCR3 (Fig. 1f) and diffuse cytoplasmic pattern of CXCR4 (Fig. 1 and k–n).

Quantitative Real-Time PCR (qPCR)

CXCR4, CXCR7 and their chemokine ligands CXCL11 and CXCL12 were detected in all samples including primary, recurrent and xenografted tumors. However, mRNA expression for CXCL9 and CXCL10 and their receptor CXCR3, was found in primary tumors and recurrences but was not detected in xenografts. Quantitative mRNA values found for CXCL9, CXCL10, CXCL11 and CXCL12, and the receptors CXCR3, CXCR4 and CXCR7 are shown in Table 2.

Fig. 1 **(a–c)** Immunoexpression of chemokine receptors in primary rhabdomyosarcomas. **a**, CXCR3 receptor (case 2), 10x. **(b–c)** chemokine receptors showing surface and a perinuclear expression (case 3), 40x **b**, CXCR4 expression. **c**, CXCR7 expression. **(d–f)** Immunoexpression of chemokines and chemokine receptors in xenografted RMS. **d,** CXCL12 chemokine expression (case 4), 20x. **e,** CXCR4 expression showing cytoplasmic and nuclear location (case 2), 40x. **f,** CXCR7 chemokine receptor expression (case 5), 40x. **(g–j)** Immunoexpression of chemokines in primary ERMS, 20x. **g**, CXCL12 (case 2). **h**, CXCL9 (case 1). **i**, CXCL10 (case 1). **j**, CXCL11 (case 3). **(k–n)**, expression of chemokines and chemokine receptors in cultured cells from ERMS: **k**, CXCL12 immunoexpression (case 1, primary tumor); **l**, CXCR3 expression, showing a granular cytoplasmic pattern (case 1, recurrence); **m–n**, CXCR4 expression in a primary ERMS and its recurrence (case 1). **(o–p)** mRNA ratios of chemokines/chemokine-receptors by qPCR. **o**, primary and recurrent tumors; $p<0.05$ for CXCL11/CXCR3 and CXCL12/CXCR4. **p,** xenografts; $p<0.05$ for CXCL12/CXCR7, CXCL12/CXCR4 and CXCL11/CXCR7. **q**, Representative flow cytometry charts of CXCR4 in cultured cells from cases 2, 3 and 4. *Blue*, surface expression of chemokine receptor CXCR4; *red*, fluorescent signal of isotypes detected. (*) Statistically significant results

We have also studied the ratio: chemokine versus the correspondent receptor, for each sample. CXCL10/CXCR3 and CXCL11/CXCR3 were lower in primary tumors than in the recurrences derived from them. Contrarily, the axes CXCL12/CXCR4, CXCL11/CXCR7, and CXCL12/CXCR7 were higher in primary tumors than in their recurrences (Fig. 1o). The differences were statistically significant between primary and recurrent RMS for CXCL11/CXCR3 and CXCL12/CXCR4 axis ($p \leq 0.05$).

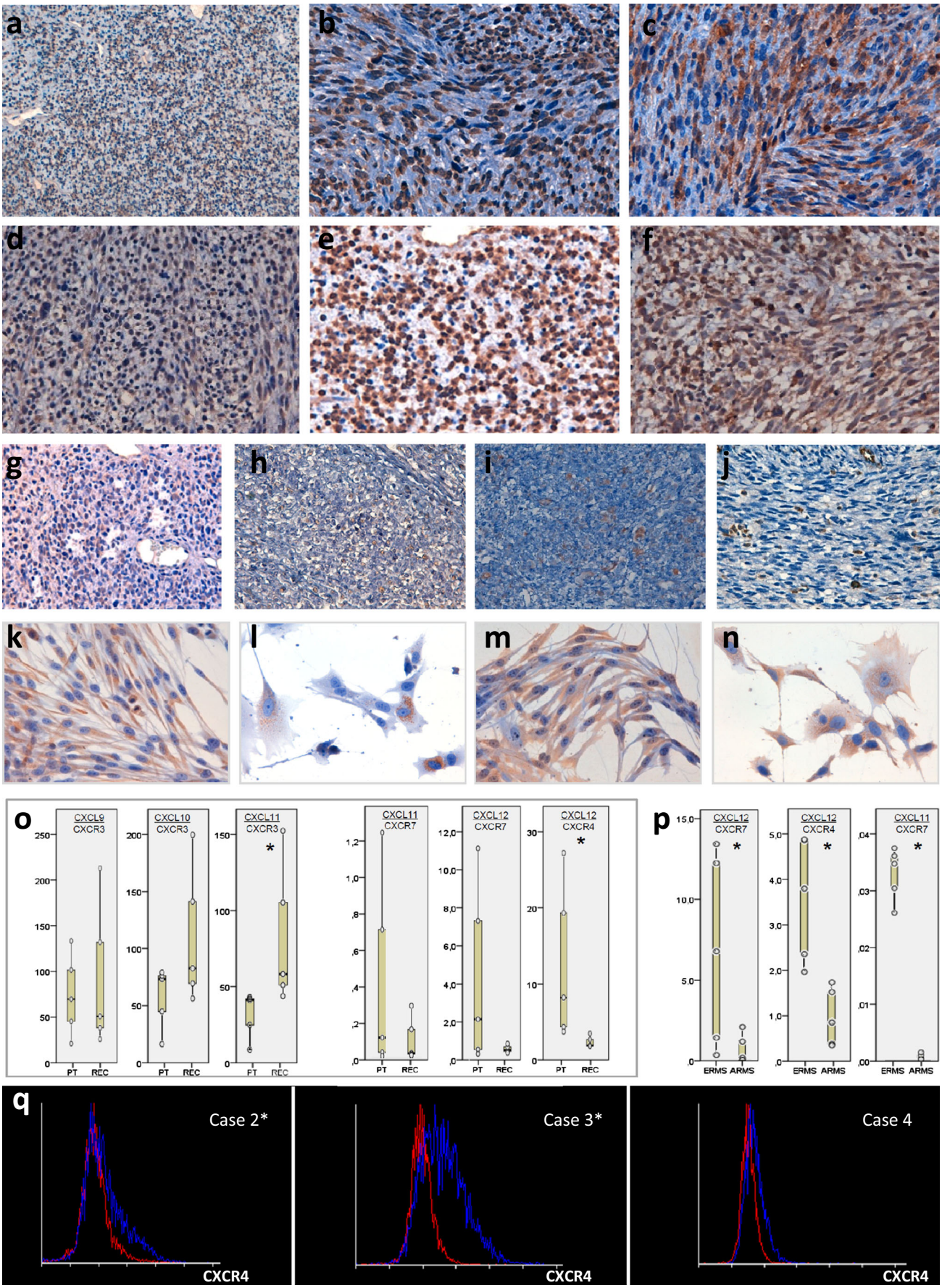
We have also studied 8 xenografts derived from RMS (3 ARMS and 5 ERMS). We analyze the axes involving CXCR4 and CXCR7, because CXCR3 and its ligands were not detected in xenografts. The ratios CXCL11/CXCR7, CXCL12/CXCR7 and CXCL12/CXCR4 were higher in ERMS than in ARMS in a significantly manner ($p \leq 0.05$) (Fig. 1p).

Flow Cytometry

Surface expression of CXCR3, CXCR4 and CXCR7 receptors was detected in cases 2 and 3 (Fig. 1q). Case 1 only showed surface expression of CXCR7. This receptor was the most widely detected at the surface of cultured RMS cells. Percentage of positive surface expression for chemokine receptors versus isotype antibodies are shown in Fig. 2. Expression of CXCL9, CXCL11 and CXCL12 chemokines was detected in all cases (Fig. 3); CXCL12 was the ligand expressed in a higher percentage of cells, with over 60 % positivity in all samples.

Discussion

Rhabdomyosarcoma is a malignant tumor that affects mainly children and adolescents. Metastasis of RMS frequently involves the lungs and bone marrow [34]. Despite the efforts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Case 4
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Table 2 mRNA expressión of chemokines and their receptors in rhabdomyosarcoma

Case	Sample	Source	CXCL9	CXCL10	CXCL11	CXCL12	CXCR3	CXCR4	CXCR7
1	1	PT	7,24E-03	5,31E-03	4,22E-03	3,83E-02	3,94E-04	8,13E-03	3,38E-03
1	2	R	3,89E-05	1,26E-04	9,50E-05	1,93E-03	1,67E-06	1,18E-03	3,47E-03
2	3	PT	4,58E-04	2,23E-04	7,15E-04	3,25E-02	0,00E+00	9,28E-03	6,52E-02
2	4	R	3,11E-05	1,19E-04	9,29E-05	4,50E-03	6,44E-07	2,67E-03	6,37E-03
2	8	XPT	0,00E+00	0,00E+00	2,81E-06	1,17E-03	0,00E+00	4,70E-04	1,08E-04
2	9	X R	0,00E+00	0,00E+00	2,53E-06	8,92E-04	4,78E-08	4,99E-04	6,76E-05
3	5	PT	1,92E-04	2,06E-04	1,22E-04	2,25E-03	2,85E-06	2,03E-04	2,33E-03
3	6	R	3,75E-03	9,05E-04	7,72E-04	2,75E-03	1,78E-05	8,46E-04	2,67E-03
4	7	PT	3,20E-03	1,63E-03	9,98E-04	2,11E-02	2,44E-05	7,94E-04	5,73E-03
4	10	XPT	0,00E+00	0,00E+00	0,00E+00	1,46E-03	0,00E+00	3,31E-04	4,51E-03
5	11	XPT	0,00E+00	0,00E+00	1,21E-05	8,45E-04	0,00E+00	1,91E-05	3,48E-04
6	12	XPT	0,00E+00	0,00E+00	1,91E-07	1,90E-04	0,00E+00	1,21E-04	7,39E-04
7	13	XPT	0,00E+00	0,00E+00	0,00E+00	3,78E-05	0,00E+00	1,17E-04	6,88E-04
8	14	XPT	0,00E+00	0,00E+00	1,34E-06	2,72E-05	0,00E+00	7,44E-05	2,85E-03
9	15	XPT	0,00E+00	0,00E+00	2,22E-07	3,01E-04	0,00E+00	2,54E-04	1,48E-04

mRNA expression ($2^{-\Delta Ct}$) of chemokines and their receptors by qPCR

PT primary tumor, *R* recurrence, *XPT* xenograft from primary tumor, *XR* xenograft from recurrence

made in the last years, survival has not been improved significantly in children with advanced disease [35].

Chemokines are a family of molecules segregated by different tissues that control migratory circulation of cells and are postulated to perform a pivotal role in cancer progression. In fact, deep research about the importance of these molecules in cancer development and progression has been reported during the last decade [14, 15]. The receptors CXCR4, CXCR7, CXCR3 and their chemo-attractive ligands CXCL9, CXCL10, CXCL11 and CXCL12 have been proposed to take part in tumor aggressiveness [21, 24–30, 36, 37].

The aim of the present study was to quantify the chemokines/chemokine-receptor axis using qPCR. Furthermore we applied flow cytometric analysis and immunostaining for location of these molecules in RMS cells. In this work we found that both primary and recurrent ERMS tumors expressed all receptors (CXCR3, CXCR4 and CXCR7) and their chemokine ligands. Tumoral tissues showed mostly a surface and granular cytoplasmic expression for all chemokine receptors analyzed. CXCR4 and CXCR7 were also expressed in perinuclear site and CXCR4 also showed nuclear expression. Nuclear expression of CXCR4 has been

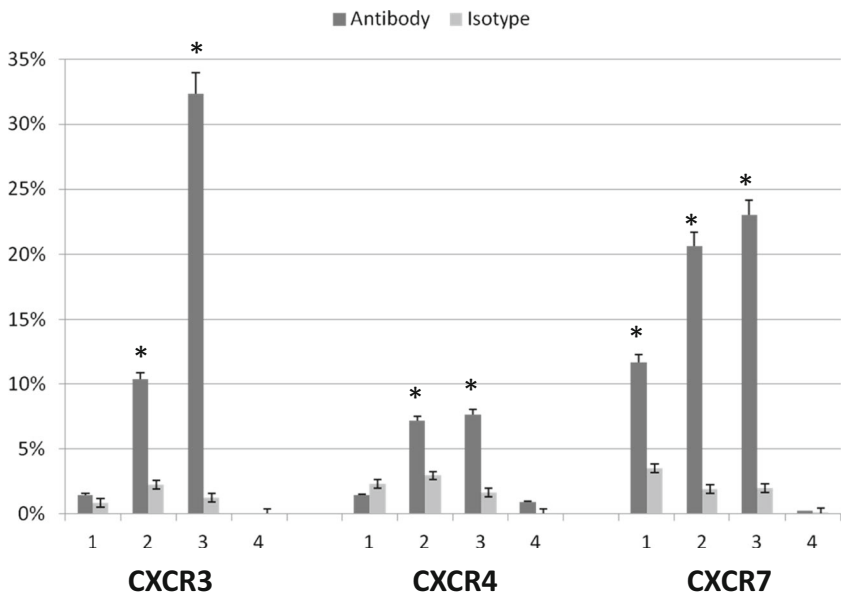

Fig. 2 Flow cytometry of chemokine receptors versus isotype antibodies. Percentage of positive cells from RMS cells in culture conditions and comparison with isotypes are represented. Flow cytometry reveals surface expression for each chemokine receptor assayed. Rhabdomyosarcoma cultured cells (cases 2 and 3) presented all the receptors analyzed (CXCR3, CXCR4 and CXCR7), case 1 was positive only for CXCR7 while case 4 did not express these receptors on the cells surface. (*) Statistically significant difference $p<0.05$

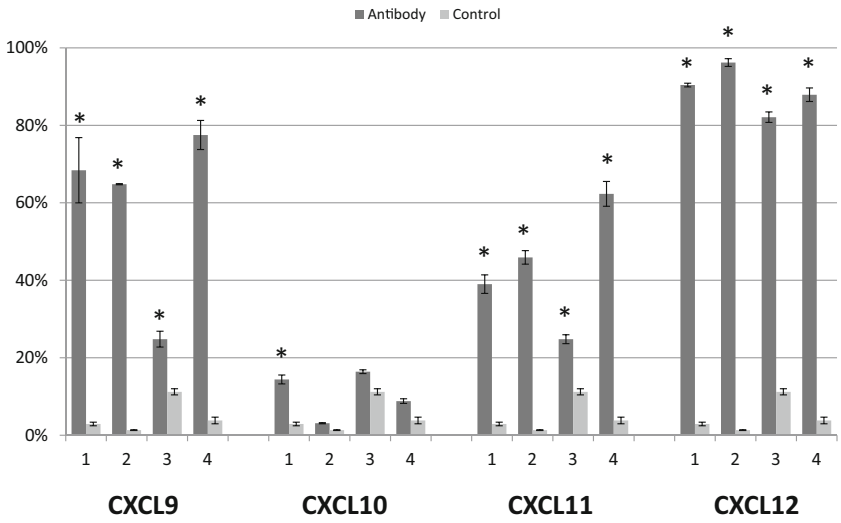

Fig. 3 Flow cytometry of chemokines CXCL9, CXCL10 and CXCL11 in RMS cells in culture. CXCL12 was the chemokine expressed in a higher percentage of cells in all the cases. (*) Statistically significant difference $p<0.05$

previously reported in other tumoral types as rectal and gastric cancer [38, 39]. Tumoral populations are very heterogeneous making the behavior of each cell different, so the number of cells with nuclear CXCR4 can vary in each case. Our group of RMS does not usually show more than 10 % of nuclear positivity of CXCR4 receptor. The percentage of tumoral cells showing this location for CXCR4 varies depending on the tumor type and the grade; nuclear expression of CXCR4 has been found in 30 % of cells in rectal tumors of stage IV [40]. High immunoreactivity of nuclear CXCR4 in gastric cancer suggests that CXCL12 binds to the CXCR4 receptor at the membrane, it translocates to the nucleus leading to a more invasive behavior and thus can be considered a prognostic factor [38]. Furthermore, CXCR4, CXCR7, CXCL11, and CXCL12 expression was detected in xenografted ERMS and ARMS. We also found expression of CXCL11 and CXCL12 in short term cultures of RMS, in contrast with the results obtained by Libura et al. [24] that find expression of CXCL12 in only a 60 % of their analyzed RMS cell lines and Grymula et al. [30], which refer no expression of CXCL11 or CXCL12 in their studies of RMS cell lines. We attribute this fact to the different types of cell cultures, as ours correspond to the first passages obtained directly from primary tumors, instead of from established RMS cell lines.

The chemokine ligand/receptor ratio has evidenced to be an excellent method for assessing chemokines available in a given tissue. In addition, this method is independent of the housekeeping gene selected for normalization, what contributes to a better standardization of the results [31, 41]. CXCR4 and CXCR7 have been reported to be higher in ARMS than in ERMS [21, 27]; in agreement with this, in the present study we found significant lower CXCL12/CXCR7, CXCL11/CXCR7 and CXCL12/CXCR4 ratios in ARMS than in ERMS xenografts. These results are equivalent to those reported in melanoma by Monteagudo et al. [31] that found the lower ratio values in the most metastatic melanomas. In the same way, we found a significantly higher CXCL12/CXCR4 ratio in primary ERMS than in their recurrences.

Variable surface expression of CXCR3, CXCR4 and CXCR7 was found in cultured cells of ERMS by cytometry (cases 1 to 4) being CXCR7 the receptor more generally expressed. Similar results have been previously reported [29, 30]. This is an interesting fact that could be implicated in malignant progression of ERMS. Expression of chemokine receptors was found also using immunoexpression techniques: We found a surface expression of CXCR3 in both RMS tumors and cultured cells, and a surface and diffuse expression for CXCR4 and CXCR7, with expression also in perinuclear and membranous sites. Finally, a small percentage of cells (10 %) showed nuclear expression of CXCR4. Nuclear localization of CXCR4 has been related with poor prognosis and/or metastasis in other tumors as colorectal cancer [42, 43], renal cell carcinoma [44] and non-small cell lung cancer [45], although its biological significance has not yet been elucidated.

In conclusion, chemokines CXCL11, CXCL12 and their receptors CXCR4 and CXCR7 were detected, by qPCR, in all analyzed samples. Interestingly, xenografted RMS do not show CXCL9, CXCL10 and their receptor CXCR3. Further studies are necessary to explain the absence of these molecules in xenografts. The lower ratios involving CXCR4 or CXCR7 seem to be related with more aggressive forms of RMS. (ERMS recurrences versus primary tumors present similar results as those of ARMS versus ERMS).

To our knowledge no comparison between the chemokine and chemokine receptors values in primary and recurrent ERMS tumors has been previously reported, as well as the chemokine expression in fresh RMS. As chemokine/chemokine-receptors ratios seem to be related with the more aggressive forms of RMS, further research in the quantification of these molecules in RMS could be important to deepen in the knowledge of mechanisms involved in tumoral progression.

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Conflict of Interest The authors declare that there are no conflicts of interest

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