FACULTAD DE MEDICINA

OSTEOSARCOMA EN LA INFANCIA Y ADOLESCENCIA: FACTORES PRONÓSTICOS E IDENTIFICACIÓN DE NUEVAS DIANAS TERAPÉUTICAS

Tesis doctoral para optar al título de
Doctor por la Universidad de Valencia con MENCION INTERNACIONAL

Pablo Berlanga Charriel

Dirigida por:

Dra. María Adela Cañete Nieto

Dr. Jaime Font de Mora Sainz

Valencia- Mayo 2017

Facultad de Medicina y Odontología

Hospital Universitari i Politècnic La Fe
Instituto de Investigación Sanitaria La Fe

D. JAIME FONT DE MORA SAINZ, DOCTOR EN CIENCIAS QUÍMICAS POR LA UNIVERSIDAD DE VALENCIA. INVESTIGADOR JEFE DEL LABORATORIO DE BIOLOGÍA CELULAR Y MOLECULAR DEL GRUPO ACREDITADO DE INVESTIGACIÓN CLÍNICA Y TRASLACIONAL EN CÁNCER DEL INSTITUTO DE INVESTIGACIÓN SANITARIA LA FE

Y

DÑA. MARIA ADELA CAÑETE NIETO, DOCTORA EN MEDICINA POR LA UNIVERSIDAD DE VALENCIA, PROFESORA ASOCIADA DEL DEPARTAMENTO DE PEDIATRÍA, OBSTETRICIA Y GINECOLOGÍA DE LA FACULTAD DE MEDICINA DE LA UNIVERSIDAD DE VALENCIA Y JEFA DE SECCIÓN DE ONCOLOGÍA PEDIÁTRICA DEL HOSPITAL LA FE

CERTIFICAN:

Que la presente Tesis Doctoral titulada: **Osteosarcoma en la infancia y adolescencia: factores pronóstico e identificación de nuevas dianas terapéuticas**, ha sido realizada favorablemente por D. Pablo Berlanga Charriel, Licenciado en Medicina por la Universidad de Valencia, bajo nuestra dirección en la Sección de Oncología Pediátrica del Hospital La Fe y en el Laboratorio de Biología Celular y Molecular del Instituto de Investigación Sanitaria La Fe.

Dicho trabajo está concluido y, en nuestro criterio, reúne todos los méritos necesarios para optar al Grado de Doctor CON MENCIÓN INTERNACIONAL por la Universidad de Valencia.

Para que conste, en cumplimiento de la legislación, firmamos el presente certificado.

Valencia, a 22 de mayo de 2017

Fdo. Jaime Font de Mora Saínz Fdo. María Adela Cañete Nieto

AGRADECIMIENTOS

Me gustaría dedicar este trabajo a tanta gente que, aunque lo revisara múltiples veces, seguro que me dejaría a muchos sin nombrar… Por ello, me gustaría utilizar algunos fragmentos del poema "La gente que me gusta" de Mario Benedetti, que resumen el tipo de GENTE a la que le estoy tan agradecido y a la que dedico este trabajo…

Gracias a "*la gente que vibra, que no hay que empujarla, que no hay que decirle que haga las cosas, sino que sabe lo que hay que hacer y que lo hace. La gente que cultiva sus sueños hasta que esos sueños se apoderan de su propia realidad*". Gracias a *"la gente con capacidad para asumir las consecuencias de sus acciones, la gente que arriesga lo cierto por lo incierto para ir detrás de un sueño*".

Gracias a *"la gente capaz de criticarme constructivamente y de frente, pero sin lastimarme ni herirme. La gente que tiene tacto"*.

Gracias a *"la gente que sabe la importancia de la alegría y la predica. La gente que mediante bromas nos enseña a concebir la vida con humor"*. Gracias a *"la gente que con su energía, contagia"*.

Gracias a *"la gente sincera y franca, capaz de oponerse con argumentos razonables a las decisiones de cualquiera"*. Gracias a *"la gente fiel y persistente, que no desfallece cuando de alcanzar objetivos e ideas se trata"*.

Gracias a *"la gente que busca soluciones"*.

Mis más sinceros agradecimientos a tanta y tanta GENTE.

RESUMEN

OSTEOSARCOMA IN CHILDREN AND ADOLESCENTS: PROGNOSTIC FACTORS AND NEW THERAPEUTIC TARGETS

Pablo Berlanga Charriel

Programa de Doctorado en Medicina, Departamento de Pediatría, Obstetricia y Ginecología, Facultad de Medicina y Odontología, Universidad de Valencia.

Introduction

Osteosarcoma is the most common primary malignant bone tumour in children and adolescents. Current management of high-grade osteosarcoma consists of administration of neoadjuvant/adjuvant chemotherapy and complete surgical removal of all tumour sites. The most important prognostic factors for long-term survival are: disease extension (localized/metastatic), primary tumour location (extremity/axial), histologic response to first-line induction chemotherapy and surgical removal of all detectable lesions.

The addition of chemotherapy to surgery in the 1970s and 80s increased substantially long-term survival of osteosarcoma patients. However, no further improvement of survival has been observed since then, with currently 5-year overall survival of 60-70% for localized and 20-30% for metastatic osteosarcoma. Therefore, there is an urgent need for new therapeutic agents to improve prognosis for osteosarcoma patients.

General objective:

- To identify prognostic factors, at diagnosis and relapse, for children and adolescents with high-grade osteosarcoma and identify new therapeutic targets.

Specific objectives:

- To identify prognostic factors and survival of children and adolescents with high-grade osteosarcoma at diagnosis and relapse.
- To determine the incidence and cancer distribution, treatment setting and provider specialty of children and adolescents in the Comunidad Valenciana.
- To identify new therapeutic targets in paediatric and adolescent osteosarcoma

Methods:

This PhD thesis consists of four papers of which the candidate is the first author:

1. **Pablo Berlanga**, Adela Cañete, Roberto Díaz, Marta Salom, Francisco Baixauli, Jacinto Gómez, Margarita Llavador, Victoria Castel. Presentation and Long-term Outcome of High-grade Osteosarcoma: A Single-institution Experience. J Pediatric Hematol Oncol 2015; 37: e272-e277.
2. **Pablo Berlanga**, Adela Cañete, Marta Salom, Joaquin Montalar, María Guasp, Alfredo Marco, Victoria Castel. Postrelapse Prognostic Factors in Nonmetastatic Osteosarcoma: A Single-Institution Experience. J Pediatric Hematol Oncol 2016; 38: 176-181.
3. **Pablo Berlanga**, María Luisa Vicente, Adela Cañete, Carmen Alberich, Victoria Castel. Cancer in cancer in children and adolescents in Spain: incidence, treatment setting and provider specialty. Clin Transl Oncol 2016; 18: 27-32.
4. **Pablo Berlanga,** Lisandra Muñoz, Marta Piqueras, Antoni Sirerol, María Dolors Sánchez-Izquierdo, David Hervás, Miguel Hernández, Margarita Llavador, Isidro Machado, Antonio Llombart-Bosch, Adela Cañete, Victoria Castel, Jaime Font de Mora. miR-200c and Akt predict osteosarcoma progression and lung metastasis. Mol Oncol. 2016; 10: 1043-53.

This work was supported by a Predoctoral Research Fellowship from the Instituto de Investigación Sanitaria La Fe (2011/0319)

Results and conclusions:

Five-year overall survival (OS), event-free survival (EFS) and main prognostic factors for high-grade osteosarcoma patients in our cohort are similar to those previously described: A) At diagnosis: metastatic disease, poor histologic response to first-line induction chemotherapy and incomplete surgical removal of all detectable lesions are the main negative prognostic factors. B) At first relapse: good histologic response to neoadjuvant first-line chemotherapy and complete surgical removal of all lesions at relapse are the main favourable prognostic factors.

Chemotherapy and surgery are the cornerstone of osteosarcoma treatment. Despite the important role of chemotherapy in the treatment of metastatic disease,

surgical removal of all tumour lesions at first-line treatment and relapse is necessary for long-term survival. The improvement of imaging techniques, such as the computed tomography (TC) scan, has increased its sensitivity while decreasing its specificity in the identification of pulmonary metastases and a new definition of "pulmonary metastases" at diagnosis with current imaging techniques is needed. In order to maintain chemotherapy intensity, a delay in the resumption of chemotherapy after primary tumour surgery needs to be avoided.

Osteosarcoma incidence is higher during adolescence. Adolescents are a unique group, with patterns of disease and healthcare challenges distinctly different than those faced by younger children and adults. In our region, childhood and adolescent cancer incidence is similar to other European countries, with higher overall incidence of malignancy in adolescents than children. Of importance, our results show an important dispersion of treatment of adolescents compared to children in the Comunidad Valenciana. We suggest the centralization of care of adolescents with cancer with the creation of specific teenager and young adult cancer centres in which these patients can benefit from the shared expertise of medical and paediatric specialists.

There is an urgent need for new therapeutic agents to improve prognosis for osteosarcoma patients. PI3K/AKT/mTOR activation, determined by phopho-AKT immunostaining, is associated with lower overall survival in osteosarcoma primary tumours. MiR-200 is overexpressed in lung metastases and plays a role in the molecular processes of lung metastasis. Therefore, PI3k/AKT/mTOR and miR-200c inhibitors are potential therapeutic targets to prevent progression and metastasis of paediatric osteosarcomas.

ÍNDICE

ABREVIATURAS

EMA: Agencia Europea del Medicamento

FDA: Food and Drud Administration

GEIS: Grupo Español de Investigación en Sarcomas

IC: Intervalo de confianza

ICCC: International Classification of Childhood Cancer

MAP: Metotrexato altas dosis, adriamicina, cisplatino

MAPIE: Metotrexato altas dosis, adriamicina, cisplatino, ifosfamida, etopósido

RC: Respuesta completa

RETI-SEHOP: Registro Español de Tumores Infantiles-SEHOP

RM: Resonancia magnética

RTICV: Registro de Tumores Infantiles de la Comunidad Valenciana

SEHOP: Sociedad Española de Oncología y Hematología Pediátricas

SEOP: Sociedad Española de Oncología Pediátrica

SG: Supervivencia global

SLE: Supervivencia libre de eventos

TC: Tomografía computarizada

TMAs: *Tissue microarrays*

COMPENDIO DE PUBLICACIONES

La presente tesis se basa en el compendio de cuatro publicaciones en las que el candidato al título de Doctor es el primer autor.

La realización de esta Tesis ha sido posible gracias a la concesión de un Contrato de Investigación Pre-doctoral del Instituto de Investigación Sanitaria La Fe para la realización del proyecto "¿Cómo integrar la biología del tumor en la terapéutica? Estudio de la activación de PI3K/Akt/mTOR en sarcomas óseos" (2011/0319).

Los detalles de las publicaciones así como los artículos completos aparecen a continuación:

ARTÍCULO I

Presentation and Long-term Outcome of High-grade Osteosarcoma: A Single-institution Experience.

Pablo Berlanga, Adela Cañete, Roberto Díaz, Marta Salom, Francisco Baixauli, Jacinto Gómez, Margarita Llavador, Victoria Castel.

J Pediatric Hematol Oncol 2015; 37: e272-277.
Impact Factor 2015: 1.146

Presentation and Long-term Outcome of High-grade Osteosarcoma: A Single-institution Experience

Pablo Berlanga, MD,† Adela Cañete, MD, PhD,* Roberto Díaz, MD,‡ Marta Salom, MD, PhD,§ Francisco Baixauli, MD,‖ Jacinto Gómez, MD,¶ Margarita Llavador, MD,# and Victoria Castel, MD, PhD*†*

Purpose: To evaluate clinicopathologic characteristics, prognostic factors, and treatment outcome of pediatric/adolescent high-grade osteosarcoma patients.

Methods/Patients: Retrospective evaluation of patients 21 years of age or younger with newly diagnosed high-grade osteosarcoma treated in a single institution. Effects of variables on event-free survival and overall survival (OS) were determined by using Kaplan-Meier survival analysis. Variables found to be significant were evaluated with multivariable Cox regression analysis.

Results: Seventy-seven patients diagnosed between January 1985 and December 2011 were included. Median follow-up time was 11.0 years (range, 1.6 to 26.4 y). Event-free survival at 5 and 10 years was 38% ± 11% and 38% ± 11%, respectively. OS at 5 and 10 years was 51% ± 12% and 45% ± 12%, respectively. Metastatic disease, prolonged time interval to resumption of chemotherapy, lower tumor necrosis rate, and lack of achievement of complete response at the end of first-line chemotherapy treatment were associated with inferior OS probabilities in univariate analysis. Upon multivariate analysis, only achievement of complete response at the end of first-line chemotherapy and tumor necrosis rate retained independent prognostic significance.

Conclusions: Prognostic factors and long-term survival are similar to those previously described. Reduction of global time interval to resumption of chemotherapy as well as a more specific and validated definition of pulmonary metastases at diagnosis are needed.

Key Words: osteosarcoma, prognostic factors, outcome, children, adolescent

(*J Pediatr Hematol Oncol* 2015;37:e272–e277)

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents younger than 20 years of age with an incidence rate of 5.0/y per million in this group of age. Histologically, osteosarcoma originates from the primitive bone forming mesenchymal cells.[1] It arises mostly from metaphysis of long bones and most commonly around the knee. Approximately 15% to 20% of patients have metastatic disease at the time of diagnosis, with lung as the most common metastatic site.[2,3] Before the development of effective combination chemotherapy regimens, only 5% to 20% of patients were cured with surgery alone. Current management of high-grade osteosarcoma with administration of neoadjuvant and adjuvant chemotherapy with at least 2 of the 4 known active drugs (doxorubicin, methotrexate, cisplatin, ifosfamide) and complete surgical removal of all tumor sites has improved cure rates up to 60% to 70% for patients with localized disease,[4–6] but only approximately to 20% to 30% for patients with primary metastatic disease.[2,3]

In this study we present a retrospective evaluation of clinicopathologic characteristics, prognostic factors, and treatment outcome of all patients 21 years of age or younger with newly diagnosed high-grade osteosarcoma treated in a single institution between 1985 and 2011.

PATIENTS AND METHODS

All consecutive patients 21 years of age or younger with histologically proven high-grade osteosarcoma treated at the Pediatric Oncology Unit or the Medical Oncology Department of the University Hospital La Fe, Valencia (Spain) from January 1985 to December 2011 were included. Follow-up data were obtained until July 2013. Patients were identified through the institutional database. Those with non–high-grade osteosarcoma, radio-induced osteosarcoma, cases referred only for consultation/surgery, those treated before referral to our institution, or those with follow-up $\leq$18 months were excluded. All pathologic cases were retrospectively reviewed to confirm diagnosis. Primary tumor was assessed by conventional radiographs and computed tomography (CT) or magnetic resonance imaging, whereas metastatic involvement was assessed by radioisotope bone scan and CT of the chest. Primary pulmonary nodules were prospectively considered as lung metastases in case of $\geq$3 nodules measuring 5 mm or a single nodule $\geq$10 mm. Retrospectively, any synchronous pulmonary nodule with unequivocal confirmation either by surgery or by progression was considered as metastatic independent of size and number.

Treatment Details

Patients were treated according to different protocols: modified T-10 protocol,[7,8] SFOP OS87,[9] Spanish Society of Pediatric Oncology protocols (SEOP-SO-95,[10] SEOP-SO-MP-2000, SEOP-SO-2001, SEHOP-SO-2010), and MAP.[11] Preoperative and postoperative chemotherapy was given to all patients. All protocols included high-dose methotrexate

Received for publication April 8, 2014; accepted September 4, 2014.

From the *Pediatric Oncology Unit; ‡Medical Oncology Department; §Pediatric Orthopedics Unit; ‖Orthopedics Department; ¶Pediatric Radiology Unit; #Pathology Department; and †Cancer Clinical and Translational Research Group, Health Research Institute La Fe, Valencia, Spain.

P.B. is supported by a grant of the Instituto de Investigación La Fe (2011/0319).

The authors declare no conflict of interest.

Reprints: Pablo Berlanga, MD, Pediatric Oncology Unit, Hospital La Fe, Bulevar Sur s/n, 46026, Valencia, Spain (e-mail: berlanga_pab@gva.es).

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Website, www.jpho-online.com.

at 8 to 12 g/m^2 per course with leucovorin rescue, doxorubicin 75 to 90 mg/m^2 per course, and cisplatin 100 to 120 mg/m^2 per course. All protocols, except for MAP and the modified T-10, included ifosfamide 6 to 9 g/m^2 per course. In addition, bleomycin, cyclophosphamide, etoposide, and dactinomycin were used in varying combinations (Supplemental data table, Supplemental Digital Content, http://links.lww.com/JPHO/A82). The scheduled duration of chemotherapy ranged from 34 to 42 weeks. Definitive surgery was scheduled to take place between weeks 6 and 16 with attempted complete removal of the tumor with wide or radical surgical margins and limb-sparing surgery when possible. All primary metastases were also removed surgically if feasible. Time interval between last course of neoadjuvant chemotherapy and resumption of adjuvant chemotherapy (time interval to resumption of chemotherapy) was planned no longer than 3 to 4 weeks.

Clinicopathologic variables recorded and analyzed included date of birth/diagnosis/surgery/relapse/death/last contact, sex, tumor location, presence of metastasis at the time of diagnosis, symptoms at presentation, duration of symptoms, histopathologic subtype of tumor, chemotherapy regimen, type of local treatment, surgical margins, tumor response to neoadjuvant chemotherapy (tumor necrosis rate), time since last neoadjuvant chemotherapy cycle to definitive surgery, time to resumption of chemotherapy after definitive surgery, achievement of complete response (CR) at the end of first-line chemotherapy, and presence of relapse. The date of diagnosis was defined as the date of initial biopsy. CR was defined as disappearance of macroscopic tumor at all sites of disease. Relapse was defined as new appearance of disease after achievement of CR after first-line therapy. The date of relapse was defined as the date of the histologic confirmation or radiologic imaging for patients who did not undergo biopsy or tumor resection at relapse. Time interval to resumption of chemotherapy was defined as the time since last neoadjuvant chemotherapy to the first adjuvant chemotherapy. Overall survival time (OS) was measured from the date of initial diagnosis to the date of death or last contact. Event-free survival time (EFS) was defined as the minimum time from initial diagnosis to the date of death, relapse, progressive disease, or last contact.

Statistical Analysis

Categorical variables were described with the numerical count (percentage) of each category. Continuous variables were described as mean value ± SD if the distribution was normal ($P > 0.05$, Kolmogorov-Smirnov) or as median and range value if the distribution was not normal. Effects of variables on EFS and OS were determined by using Kaplan-Meier survival analysis and Log rank test, and 95% confidence intervals were calculated. Variables found to be significant were evaluated with multivariable Cox regression analysis. $P < 0.05$ was considered as statistically significant. Data were analyzed using R 3.0.2.

RESULTS

Patients

A total of 85 patients with high-grade osteosarcoma were treated at our institution during this 27-year period. Eight of them were excluded from this report because they were lost to follow-up (6) and because osteosarcoma was secondary to radiation (2). Of the 77 patients with high-grade osteosarcoma who were diagnosed from January 1985 to December 2011, there were 2 patients with osteosarcoma as second non–radio-induced malignancy (acute lymphoblastic leukemia and unilateral retinoblastoma treated 6 and 8 y previously). Median age at diagnosis was 12.9 years (range, 1.9 to 21.3 y), with only 3 patients diagnosed in the first 5 years of life. Forty-two patients (54.5%) were male and 35 patients (45.5%) female. Nineteen patients (25%) presented synchronous pulmonary nodules; however, only 10 of them (13%) were initially considered metastatic according to the nodules' size and number (≥ 3 nodules measuring 5 mm or a single nodule ≥ 10 mm). Only 1 patient presented metastatic bone involvement at diagnosis. Most of the tumors (92%) were located in the extremities and the most common histopathologic type was osteoblastic. Median symptomatic time to diagnosis was 8 weeks (range, 0 to 100 wk) and pain and/or swelling were the most common symptoms. Patients' characteristics are summarized in Table 1.

Chemotherapy

Patients were treated during this 27-year period at our hospital according to different protocols that included high-dose methotrexate, doxorubicin, and cisplatin (Table 1). Forty-three patients (56%) were treated at the Pediatric Oncology Unit, whereas 34 at the Medical Oncology Department.

Surgery and Histologic Response Assessment

Primary tumor was resected in 68 patients (88%). Nine patients did not undergo primary tumor resection because of inoperable primary tumors (3 patients with pelvic location) and early metastatic progression before local surgery in 6 patients (5 of them already metastatic at diagnosis). Primary surgery type was limb sparing in 52 patients (77%) and amputation in 16 (23%). Surgical margins were considered adequate (radical or wide) in 65 patients and marginal/intralesional in 3 patients. Five patients underwent directly local surgery due to previous histologic misdiagnosis. Therefore, histologic response assessment was only available in 63 of the 77 patients. Information on response was available for 59 of 63 tumors operated after neoadjuvant chemotherapy. Median tumor response to neoadjuvant chemotherapy was 60% (range, 0% to 100%), with no difference between localized and metastatic patients, and only 19 patients (32%) achieved a good response (necrosis ≥ 90%). Time interval to resumption of chemotherapy was 51 days (range, 26 to 139 d).

OS and EFS Analysis

Median follow-up time for survivors was 11.0 years (range, 1.6 to 26.4 y). During the follow-up period, 46 patients (60%) had recurrent/progressive disease. EFS at 2, 5, and 10 years were 50% ± 11%, 38% ± 11%, and 38% ± 11%, respectively. OS at 2, 5, and 10 years was 61% ± 11%, 51% ± 12%, and 45% ± 12%, respectively. Of the 40 deceased patients, 39 died due to disease progression and 1 patient due to toxic death. Cumulative EFS and OS curves are shown in Figure 1.

Of the 10 patients initially considered metastatic according to the nodules' size and number, in all of them the presence of pulmonary metastases was confirmed either by surgery or by progression. Concerning the remaining 9 patients with synchronous pulmonary nodules not considered initially as metastatic, 5 were confirmed as metastases (4 by surgery and 1 by progression), whereas in 2 cases nodules

TABLE 1. Patients' Characteristics (n = 77)

Characteristic	n (%)
Sex	
Male	42 (54.5)
Female	35 (45.5)
Site of primary disease	
Femur	44 (57.1)
Tibia	18 (23.4)
Humerus	6 (7.8)
Fibula	3 (3.9)
Pelvis	3 (3.9)
Other	3 (3.9)
Histology	
Osteoblastic	19 (45.2)
Chondroblastic	8 (19.0)
Telangiectatic	6 (14.3)
Fibroblastic	5 (11.9)
Other	4 (9.5)
Symptom at diagnosis	
Pain	69 (89.6)
Swelling	55 (71.4)
Pain + swelling	51 (66.2)
Protocol treatment	
Modified T-10	26 (33.8)
SFOP OS87	11 (14.3)
SEOP-SO-95	7 (9.1)
SEOP-SO-MP-2000	2 (2.6)
SEOP-SO-2001	11 (14.3)
SEHOP-SO-2010	4 (5.2)
MAP	11 (14.3)
Other	5 (6.5)
Treatment Unit	
Pediatric Oncology Unit	43 (55.8)
Medical Oncology Department	34 (44.2)
Primary metastatic disease at diagnosis	10 (13.0)
Lung	9 (11.7)
Lung + bone	1 (1.3)

resolved with chemotherapy (and did not relapse afterward) and complete surgical removal in 2 patients showed no metastatic disease. Therefore, finally 15 patients (19%) were retrospectively considered metastatic with a 5-year OS and EFS of 10% ± 10%, compared to the nonmetastatic patients with a 5-year OS and EFS of 62% ± 12% and 57% ± 12% ($P = 0.000$).

Univariate analysis of prognostic factors for OS showed no correlation between survival and age, sex, tumor location, duration of prediagnostic symptoms, treatment Unit, type of surgery, or period of treatment. Presence of metastases at diagnosis ($P = 0.001$), prolonged time interval to resumption of chemotherapy ($P = 0.021$), lower tumor necrosis rate ($P = 0.004$), and lack of achievement of CR at the end of first-line chemotherapy treatment ($P = 0.000$) were associated with inferior OS probabilities in univariate analysis (Table 2).

Pulmonary metastatic disease at diagnosis (defined as any synchronous pulmonary nodules with unequivocal confirmation either by surgery or by progression, independent of size and number) was clearly associated with inferior OS probability. Among the 15 patients considered metastatic according to this definition, only 2 survived (1.8 and 7.0 y of follow-up), whereas 35 patients of the 62 patients who presented with localized disease are survivors with a median follow-up time of 11.0 years (range, 1.6 to 26.4 y) ($P = 0.001$).

Achievement of CR at the end of first-line chemotherapy treatment was a highly significant predictor of outcome, and none of the patients with partial response/stable disease/progression at that moment are long-term survivors ($P = 0.000$).

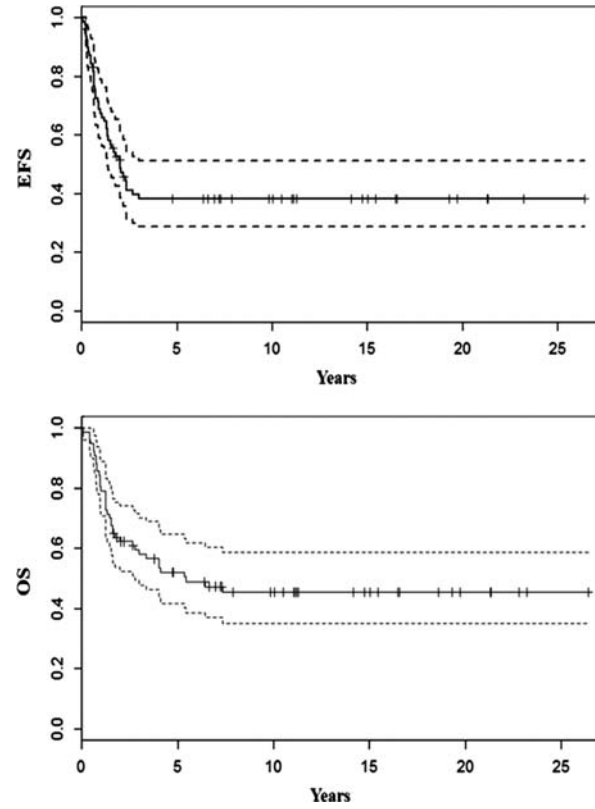

FIGURE 1. EFS and OS for all patients (95% confidence interval) (n = 77).

Prolonged time interval to resumption of chemotherapy was also associated with inferior OS probability with a median of 50 days (range, 26 to 77 d) for survivors compared with 63 days (range, 26 to 139 d) for the deceased group ($P = 0.021$).

Tumor necrosis rate after neoadjuvant chemotherapy was also correlated with outcome, with a median tumor necrosis rate of 80% (range, 10% to 100%) for survivors compared with 30% (range, 0% to 100%) for nonsurvivors ($P = 0.004$).

Multivariate analysis of prognostic factors for OS was performed with the 4 factors that had shown correlation with survival in univariate analysis. Of the 63 patients who had been operated after neoadjuvant chemotherapy, the histologic response assessment was complete in 59 and therefore included in the final analysis. Achievement of CR at the end of first-line chemotherapy ($P = 0.000$) and tumor necrosis rate ($P = 0.026$) were significantly associated with poor OS, whereas the presence of metastases at diagnosis and prolonged time interval to resumption of chemotherapy were not significantly associated ($P = 0.098$ and 0.41, respectively) (Table 3).

DISCUSSION

Osteosarcoma is the most frequent primary bone tumor in children and adolescents.[12] Long-term survival has increased substantially from 10% to 20% when surgery as single treatment was given before the 1980s up to 50% to 60% from 1985 onward because of the combination of

TABLE 2. Univariate Analysis of Prognostic Factors

Variable	Survivors (n = 37)	Exitus (n = 40)	*P*
Age	13.9 ± 3.7	12.7 ± 4.6	0.201
Sex (n [%])			0.180
Male	22 (59.5)	20 (50)	
Female	15 (40.5)	20 (50)	
Primary metastatic disease (n [%])			0.000
No	35 (94)	27 (68)	
Yes	2 (6)	13 (32)	
Site primary disease (n [%])			0.089
Extremity	36 (97.3)	35 (87.5)	
Axial	1 (2.7)	5 (12.5)	
Symptomatic time to diagnosis (wk)	10 (1-70)	8 (1-100)	0.435
Treatment Unit (n [%])			0.951
Pediatric Oncology	22 (59.5)	21 (52.5)	
Medical Oncology	15 (40.5)	19 (47.5)	
Time CTX-Sx (d)	24 (11-46)	24 (9-72)	0.146
Time Sx-CTX (d)	26 (15-62)	34.5 (14-122)	0.082
Time CTX-Sx-CTX (d)	50 (26-77)	63 (26-139)	0.021
Surgery (n [%])			0.203
Conservative	29 (78.4)	30 (75)	
Radical	8 (21.6)	10 (25)	
Week of surgery	14 (0-28)	13 (0-35)	0.724
Tumor necrosis rate (%)	80 (10-100)	30 (0-100)	0.004
RC at the end of first-line treatment (n [%])	37 (100)	15 (37.5)	0.000
Period of treatment (n [%])			
≤1997	19 (51.4)	21 (52.5)	0.892
> 1997	18 (48.6)	19 (47.5)	

Time CTX-Sx indicates time since last neoadjuvant chemotherapy cycle to definitive surgery; Time CTX-Sx-CTX, global time to resumption of chemotherapy; Time Sx-CTX, time to resumption of chemotherapy after definitive surgery.

multiagent chemotherapy and advanced surgery. However, since then no further substantial improvement of survival has been observed.[4,13,14] Large tumor volume, axial location, presence of metastases at time of initial diagnosis, poor histologic response to first-line induction chemotherapy, and complete surgical excision of all detectable lesions are the most important prognostic factors for long-term survival.[2,14–17]

Here, we aimed to retrospectively study management of all patients 21 years of age or younger with high-grade osteosarcoma in our institution, when modern regimens of multidrug neoadjuvant/adjuvant chemotherapy in combination with surgery were consistently included in our institution. Patients below 14 years of age are usually treated in the Pediatric Oncology Unit, whereas most of those older than 14 years are referred to the Medical Oncology Department. Low-grade variants, associated with lower likelihood of metastases, were excluded.[18]

TABLE 3. Multivariate Analysis of Prognostic Factors

Variable	Hazard Ratio	95% CI	*P*
Primary metastatic disease	3.047	0.817-11.357	0.097
Time CTX-Sx-CTX	0.999	0.979-1.019	0.913
Tumor necrosis rate	0.982	0.967-0.998	0.026
RC end first-line treatment	50.004	8.764-285.307	< 0.001

CI indicates confidence interval.

Patient characteristics (age, sex, site of primary disease, and site of metastatic involvement) are consistent with those found in the literature[1,4,14,16,17,19,20]: male/female ratio was 1.2, most of the tumors (92%) were located in the extremity (mainly femur and tibia), most were of osteoblastic subtype, and 19% presented with metastases at diagnosis.[2,3] All patients received at least 3 of the 4 known active drugs against osteosarcoma (doxorubicin, methotrexate, cisplatin, ifosfamide)[13–15] and complete surgical removal of all tumor sites was achieved when feasible with adequate surgical margins (96%), mainly through limb-salvage surgery (77%).

After a median follow-up of 11.0 years, 40 patients are alive with a 5- and 10-year survival probability of 51% ± 12% and 45% ± 12%, respectively. Several factors might explain our inferior results compared with the expected 50% to 70% 5-year survival.[1,2,4,5,15,19,20] On the one hand, in our cohort 9 patients (12%) were not operated compared with the 3% to 4% published, mainly because of inoperable primary tumors (3 pelvic tumors) and early metastatic progression before local surgery (6 patients, all them in the long bones, and 5 of them already metastatic at diagnosis). On the other hand, on the operated patients, tumor necrosis rate was lower than expected with median histologic response to first-line chemotherapy of 60% and only 19 patients (32%) classified as good responders, compared with the 50% to 60% published.[4,20,21] Histologic response to preoperative chemotherapy is one of the most important prognostic factors in osteosarcoma, and poor response (> 10% viable tumor) has consistently been associated with increased rates of local failure and metastatic recurrence.[15,21] In our cohort there was no difference in preoperative chemotherapy or in the timing of the surgery between good and poor responders. Although the most plausible explanation might be the limited number of patients involved in our study, lower tumor necrosis rate and early metastatic progression before local surgery might imply a more aggressive biological behavior of our patients' tumors.

Separate analysis according to the presence/absence of metastatic disease at diagnosis shows a 5-year OS and EFS of 62% ± 12% and 57% ± 12%, respectively, for the primary nonmetastatic patients and 10% ± 10% for the metastatic ones ($P = 0.000$). OS and EFS curves for metastatic/nonmetastatic patients at diagnosis are shown in Figure 2. However, definition of metastatic disease is variable in the literature and survival can vary depending on the definition of pulmonary metastases used. If only synchronous pulmonary nodules were considered as lung metastases in case of ≥3 nodules measuring 5 mm or a single nodule ≥10 mm, then only 10 patients (13%) would have been considered metastatic and 5-year OS would be 0%. However, in case any synchronous pulmonary nodules independent of size and number had been considered as metastatic, then 19 patients (25%) would have been considered as metastatic and 5-year OS would be 24% ± 20%.

Metastatic osteosarcoma 5-year OS is generally considered between 10% and 30%.[2,3] However, this might greatly depend on the definition of primary pulmonary metastases used. Survival of osteosarcoma patients with disease spread at diagnosis, mainly to the lung, is clearly worse than those with no evidence of osteosarcoma

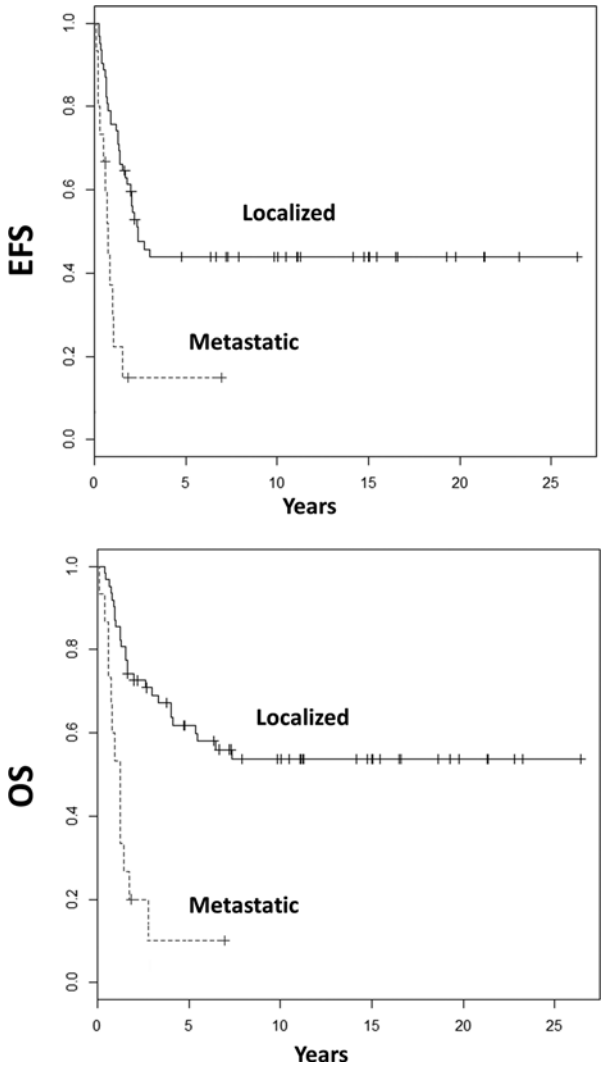

FIGURE 2. EFS and OS for localized/metastatic patients ($P = 0.001$) (n = 77).

dissemination. However, lack of homogenous definition for synchronous pulmonary metastases leads to significant differences in survival rates among different publications. Whereas most clinicians agree that one pulmonary or pleural nodule measuring 10 mm or multiple lesions >5 mm are evidence of pulmonary metastatic spread, others restrict that definition only to proven metastases by surgery or progression.[3] However, other groups consider any pulmonary nodule >5 mm as metastatic,[2] whereas for others any pulmonary nodule on chest CT independent on size is considered as a pulmonary metastasis[22] and many others simply do not specify what has been considered as lung metastases.[23–25]

The evolution of CT has improved the visualization and identification of very small pulmonary nodules that would have passed unnoticed before, and, although different features such as size, number, calcification, or growth factors have been studied as predictors of malignancy, no clear guidance in the literature exists.[26–28] In our cohort, all the 9 patients with synchronous pulmonary nodules not considered initially as metastatic due to their size and number were diagnosed after 2005: 5 of them were confirmed as metastases, whereas 4 of them were not. The improvement in CT quality has increased the sensitivity of detection of pulmonary nodules, enabling early detection of high-risk patients with nodules that would have previously considered as localized and could benefit from a more aggressive approach with surgical removal of all suspected nodules that might improve survival.

There was no correlation between survival and age, sex, tumor location, duration of prediagnostic symptoms, treatment (adult or pediatric) Unit, type of surgery, or period of treatment. Presence of metastases at diagnosis, prolonged time interval to resumption of chemotherapy, lower tumor necrosis rate, and lack of achievement of CR at the end of first-line chemotherapy treatment were associated with inferior OS probabilities in univariate analysis (Table 2). Upon multivariate analysis, achievement of CR at the end of first-line chemotherapy and tumor necrosis rate retained independent prognostic significance, whereas prolonged time interval to resumption of chemotherapy and presence of metastases at diagnosis did not.

Metastatic disease is one of the most important prognostic factors in osteosarcoma.[2,3] Of our 15 patients with metastatic disease, in only 3 of them a CR was achieved at the end of first-line chemotherapy and 2 of them are alive. If achievement of CR were not considered in the statistical model, metastatic disease would be an independent prognostic factor with a 95% confidence interval of 1.918-20.795 ($P = 0.002$) (data not shown). However, when both variables were considered in the same model, metastatic disease loses its significance probably due to the limited number of metastatic patients.

A delay in the resumption of chemotherapy after definitive surgery for the treatment of osteosarcoma can decrease the overall dose intensity, affecting the outcome. A previous COG study has shown that a delay of >21 days in the resumption of chemotherapy after definitive surgery is associated with a decrease in OS in patients with localized osteosarcoma.[29] In our study prolonged global time interval to resumption of chemotherapy was only statistically significant in the univariate analysis, but most troubling is that in 75% of the cases, resumption of chemotherapy after definitive surgery was beyond the "21-day" period and that median time interval between last neoadjuvant and first adjuvant chemotherapy cycle was of 50 to 63 days, compared with the 3 to 4 weeks recommended.

Our study has several limitations, including single-institution data, small number of patients, and long inclusion period (27 y). Because of the retrospective collection of data, information on tumor size (significant predictor of poor outcome) was not available in most patients and therefore not included in the analyses. Similarly, histopathologic type was not included as there were only data in 42/73 patients. Despite these limitations, this study represents, to our knowledge, the first published specific cohort of pediatric and adolescent Spanish osteosarcoma patients within a single institution.

In conclusion, achievement of CR at the end of first-line chemotherapy and tumor necrosis rate and the presence of metastases at diagnosis are long-term survival prognostic factors in osteosarcoma patients. Reduction of the global time interval to resumption of chemotherapy as well as a more specific and validated definition of pulmonary metastases at diagnosis are needed.

REFERENCES

1. Gurney JG, Swenson AR, Bulterys M. Malignant bone tumors. In: Ries LA, Smith MAS, Gurney JG, et al, eds. *Cancer Incidence and Survival Among Children and Adolescents: United States SEER Program 1975-1995*. Bethesda MD: SEER Program, National Cancer Institute; 1999:99.
2. Mialou V, Philip T, Kalifa C, et al. Metastatic osteosarcoma at diagnosis: prognostic factors and long-term outcome–the French pediatric experience. *Cancer*. 2005;104:1100–1109.
3. Kager L, Zoubek A, Potschger U, et al. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. *J Clin Oncol*. 2003;21:2011–2018.
4. Bielack S, Jurgens H, Jundt G, et al. Osteosarcoma: the COSS experience. *Cancer Treat Res*. 2009;152:289–308.
5. Ferrari S, Palmerini E, Staals EL, et al. The treatment of nonmetastatic high grade osteosarcoma of the extremity: review of the Italian Rizzoli experience. Impact on the future. *Cancer Treat Res*. 2009;152:275–287.
6. Jaffe N, Puri A, Gelderblom H. Osteosarcoma: evolution of treatment paradigms. *Sarcoma*. 2013;2013:203531.
7. Aparicio J, Segura A, Montalar J, et al. Long-term results after combined modality treatment for non-metastatic osteosarcoma. *Med Oncol*. 1999;16:255–260.
8. Rosen G, Caparros B, Groshen S, et al. Primary osteogenic sarcoma of the femur: a model for the use of preoperative chemotherapy in high risk malignant tumors. *Cancer Invest*. 1984;2:181–192.
9. Violas P, Kohler R, Mascard E, et al. Conservative surgical treatment of osteogenic sarcoma of the limb in children and adolescents. *Rev Chir Orthop Reparatrice Appar Mot*. 2000;86: 675–683.
10. Munoz A, Alfaro J, Pardo N, et al. Long-term results of the Spanish Protocol SO-95 for the treatment of non-metastatic high-grade osteosarcoma of the extremities in children. *Clin Transl Oncol*. 2009;11:387–392.
11. Meyers PA, Schwartz CL, Krailo M, et al. Osteosarcoma: a randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate. *J Clin Oncol*. 2005;23:2004–2011.
12. Arndt CA, Rose PS, Folpe AL, et al. Common musculoskeletal tumors of childhood and adolescence. *Mayo Clin Proc*. 2012;87:475–487.
13. Anninga JK, Gelderblom H, Fiocco M, et al. Chemotherapeutic adjuvant treatment for osteosarcoma: where do we stand? *Eur J Cancer*. 2011;47:2431–2445.
14. Ritter J, Bielack SS. Osteosarcoma. *Ann Oncol*. 2010;21(suppl 7): vii320–vii325.
15. Bielack S, Kempf-Bielack B, Von Kalle T, et al. Controversies in childhood osteosarcoma. *Minerva Pediatr*. 2013;65:125–148.
16. Bielack SS, Kempf-Bielack B, Delling G, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol*. 2002;20:776–790.
17. Whelan JS, Jinks RC, McTiernan A, et al. Survival from high-grade localised extremity osteosarcoma: combined results and prognostic factors from three European Osteosarcoma Intergroup randomised controlled trials. *Ann Oncol*. 2012;23: 1607–1616.
18. Klein MJ, Siegal GP. Osteosarcoma: anatomic and histologic variants. *Am J Clin Pathol*. 2006;125:555–581.
19. Picci P, Mercuri M, Ferrari S, et al. Survival in high-grade osteosarcoma: improvement over 21 years at a single institution. *Ann Oncol*. 2010;21:1366–1373.
20. Ferrari S, Ruggieri P, Cefalo G, et al. Neoadjuvant chemotherapy with methotrexate, cisplatin, and doxorubicin with or without ifosfamide in nonmetastatic osteosarcoma of the extremity: an Italian sarcoma group trial ISG/OS-1. *J Clin Oncol*. 2012;30:2112–2118.
21. Andreou D, Bielack SS, Carrle D, et al. The influence of tumor- and treatment-related factors on the development of local recurrence in osteosarcoma after adequate surgery. An analysis of 1355 patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. *Ann Oncol*. 2011;22: 1228–1235.
22. Kaste SC, Pratt CB, Cain AM, et al. Metastases detected at the time of diagnosis of primary pediatric extremity osteosarcoma at diagnosis: imaging features. *Cancer*. 1999;86:1602–1608.
23. Durnali A, Alkis N, Cangur S, et al. Prognostic factors for teenage and adult patients with high-grade osteosarcoma: an analysis of 240 patients. *Med Oncol*. 2013;30:624.
24. Daw NC, Billups CA, Rodriguez-Galindo C, et al. Metastatic osteosarcoma. *Cancer*. 2006;106:403–412.
25. Briccoli A, Rocca M, Salone M, et al. High grade osteosarcoma of the extremities metastatic to the lung: long-term results in 323 patients treated combining surgery and chemotherapy, 1985-2005. *Surg Oncol*. 2010;19:193–199.
26. Absalon MJ, McCarville MB, Liu T, et al. Pulmonary nodules discovered during the initial evaluation of pediatric patients with bone and soft-tissue sarcoma. *Pediatr Blood Cancer*. 2008;50:1147–1153.
27. Picci P, Vanel D, Briccoli A, et al. Computed tomography of pulmonary metastases from osteosarcoma: the less poor technique. A study of 51 patients with histological correlation. *Ann Oncol*. 2001;12:1601–1604.
28. Brader P, Abramson SJ, Price AP, et al. Do characteristics of pulmonary nodules on computed tomography in children with known osteosarcoma help distinguish whether the nodules are malignant or benign? *J Pediatr Surg*. 2011;46:729–735.
29. Imran H, Enders F, Krailo M, et al. Effect of time to resumption of chemotherapy after definitive surgery on prognosis for non-metastatic osteosarcoma. *J Bone Joint Surg Am*. 2009;91:604–612.

ARTÍCULO II

Postrelapse Prognostic Factors in Nonmetastatic Osteosarcoma: A Single-Institution Experience.

Pablo Berlanga, Adela Cañete, Marta Salom, Joaquin Montalar, María Guasp, Alfredo Marco, Victoria Castel.

J Pediatric Hematol Oncol 2016; 38: 176-181.
Impact Factor 2015: 1.146

Postrelapse Prognostic Factors in Nonmetastatic Osteosarcoma: A Single-institution Experience

Pablo Berlanga, MD, Adela Cañete, MD, PhD,* Marta Salom, MD, PhD,†*
Joaquin Montalar, MD, PhD,‡ Maria Guasp, MD,§
Alfredo Marco, MD, PhD,‖ and Victoria Castel, MD, PhD¶

Purpose: The purpose of this study was to analyze the prognostic factors that influence postrelapse survival (PRS) in children and adolescents with initial localized high-grade osteosarcoma.

Methods/Patients: This is a retrospective evaluation of patients aged 21 years and below with nonmetastatic high-grade osteosarcoma treated at our institution from 1985 to 2011 who developed recurrent disease after achievement of an initial complete response (CR). PRS and postrelapse event-free survival (PREFS) analyses were performed using the Kaplan-Meier method and log-rank test. Multivariate Cox regression analysis was used to determine which variables were independently prognostic.

Results: Thirty-one patients were included. Median age at primary diagnosis was 13.7 years (range, 1.9 to 21.0 y). Median time to first relapse was 16 months (range, 3 to 36 mo). Fourteen patients achieved a second CR (CR2) after surgery ± chemotherapy treatment. The 5-year PRS and PREFS were both 26% (95% confidence interval, 14%-49%), with a median follow-up of 99 months (range, 27 to 271 mo). Multivariate analysis showed that achievement of CR2 ($P < 0.001$) and histologic response to first-line treatment ($P = 0.02$) were significantly associated with PRS, whereas time to first relapse did not retain univariate significance.

Conclusions: Achievement of CR2 and histologic response to preoperative first-line treatment are independent survival prognostic factors in osteosarcoma recurrence.

Key Words: osteosarcoma, recurrence, prognosis, treatment outcome, child, adolescent

(*J Pediatr Hematol Oncol* 2016;38:176–181)

Osteosarcoma is the most common primary malignant bone tumor in children and young adults.[1] Current management of high-grade osteosarcoma with administration of neoadjuvant and adjuvant chemotherapy with at least 3 of the 4 known active drugs (doxorubicin, methotrexate, cisplatin, and ifosfamide) and complete surgical removal of all tumor sites has improved cure rates up to 60% to 70% for patients with localized disease.[2–4] However, despite complete response (CR) to upfront therapy, about 30% to 35% of these patients will relapse with a much lower probability of survival (15% to 30%).[5–14]

The optimal strategy for disease recurrence treatment remains undefined. Different studies show that histologic response to first-line chemotherapy (defined as $\geq 90\%$ necrosis rate), number and site of relapse, disease-free interval, and complete remission after surgery are related to prolonged survival.[5–14]

Our aim in this study is to identify factors that influence postrelapse survival (PRS) in patients with initial diagnosis of nonmetastatic high-grade osteosarcoma.

PATIENTS AND METHODS

Eligibility criteria included the following: (1) diagnosis of nonmetastatic high-grade osteosarcoma in patients aged 21 years and below; (2) first-line treatment (complete multiagent chemotherapy and surgery) between January 1985 and December 2011 at the Pediatric Oncology Unit or the Medical Oncology Department at La Fe University Hospital, Valencia, Spain; (3) development of recurrent disease after achievement of an initial CR, defined as disappearance of macroscopic tumor at all sites of disease; and (4) treatment of relapse before January 2014 at our institution.

Patients were identified through the institutional database. All pathologic samples of initial biopsy/surgery were retrospectively reviewed to confirm diagnosis. The study was approved by our Institutional Review Board. Primary tumor was assessed by conventional radiographs and computed tomography (CT) or magnetic resonance imaging; metastatic involvement was assessed by bone scintigraphy and chest CT. Routine imaging studies (primary tumor x-rays, chest CT, and bone scans) were conducted at specified intervals for at least 5 years and in suspicion of relapse.

First-line Treatment

All patients were treated according to different protocol schedules with preoperative and postoperative chemotherapy.[15] High-dose methotrexate (8 to 12 g/m^2 + leucovorin), doxorubicin, and cisplatin were administered in all of them. Most schedules also included ifosfamide. Duration of chemotherapy ranged from 34 to 42 weeks. Definitive surgery was scheduled to take place between weeks 6 and 16, with attempted complete removal of the tumor with wide or radical surgical margins and limb-sparing surgery when possible.

Relapse treatment was individualized according to relapse site and disease-free interval. In general, aggressive surgical removal of all metastases was recommended when feasible, and chemotherapy was usually reserved for

Received for publication June 8, 2015; accepted January 23, 2016.
From the *Pediatric Oncology Unit; †Pediatric Orthopedics Unit; ‡Medical Oncology Department; §Pediatric Radiology Unit; ‖Pediatric Surgery Department, Hospital La Fe; and ¶Cancer Clinical and Translational Research Group, Health Research Institute La Fe, Valencia, Spain.
P.B. was supported by a grant from the Instituto de Investigación Sanitaria La Fe (2011/0319).
The authors declare no conflict of interest.
Reprints: Pablo Berlanga, MD, Pediatric Oncology Unit, Hospital La Fe, Avd Fernando Abril Martorell, n° 106, Valencia 46026, Spain (e-mail: berlanga_pab@gva.es).

unresectable tumors or postoperative in those with short disease-free interval.

Clinicopathologic variables that were recorded and analyzed included date of birth/diagnosis/surgery/first relapse/subsequent relapses/death/last contact, sex, tumor location and presence of metastases at diagnosis, local treatment, surgical margins, tumor histologic response to neoadjuvant chemotherapy, achievement of CR during first-line chemotherapy, relapse site, and treatment after recurrence. The date of diagnosis was defined as the date of initial biopsy. Relapse was defined as new appearance of disease after achievement of CR after initial treatment with surgery and chemotherapy. The date of relapse was defined as the date of histologic confirmation or radiologic imaging for patients who did not undergo biopsy or tumor resection at relapse. Time to first relapse was defined as the interval from initial diagnostic biopsy until first disease relapse. Second CR (CR2) was defined as macroscopically complete removal of all tumor sites. PRS was measured from the date of first relapse to the date of death or last contact. Post-relapse event-free survival (PREFS) was defined as the minimum time from first relapse to the date of progressive disease, second relapse, death, or last contact, whichever occurred first. Patients who never achieved a CR2 were considered to have a PREFS of 0 months.

Statistical Analysis

Categorical variables were described with the numerical count (percentage) of each category. Continuous variables were described as mean value ± SD if the distribution was normal ($P > 0.05$, Kolmogorov-Smirnov) or as median and range value if the distribution was not normal. PRS and PREFS analyses were performed using the Kaplan-Meier method and log-rank test. Multivariate Cox regression analysis was used to determine which variables were independently prognostic. Variables that had significance level $P < 0.1$ in the univariate analysis were included in the multivariate model. $P < 0.05$ was considered in the multivariate model as statistically significant. Data were analyzed using R 3.0.2.

RESULTS

Patient Characteristics

During the period of time of the study, 67 patients aged 21 years and below with nonmetastatic osteosarcoma were diagnosed and treated at our institution. Sixty-one of them (91%) achieved a CR after first-line treatment, and 31 met the study criteria and were further included in the current analysis. Table 1 summarizes clinical characteristics of patients at primary diagnosis and first relapse. Median age at primary diagnosis and first relapse was 13.7 years (range, 1.9 to 21.0 y) and 14.9 years (range, 2.8 to 23.0 y), respectively. Primary tumor was mainly located in the extremities (90%) and primary surgery limb sparing (84%). Histologic response to neoadjuvant chemotherapy was available in 26 patients (3 patients underwent directly local surgery because of previous histologic misdiagnosis; in 2 cases, histologic response was not specified). Median histologic response was 50% (range, 0% to 100%). Only 4 out of the 26 patients (15%) had a histologic response ≥90%.

Characteristics at First Relapse

Median time from diagnosis to first relapse was 16 months (range, 3 to 36 mo). Eleven patients (35%) relapsed during adjuvant chemotherapy treatment, whereas 20 patients (65%) relapsed after treatment. Distant relapse occurred in 20 patients (65%), mainly exclusively to the lung (16 patients); 7 patients experienced an isolated local recurrence, and the remaining 4 patients had simultaneous local and metastatic relapse. The most common site of relapse was the lung, mostly bilateral with ≥3 pulmonary nodules (Table 1). Isolated local recurrence occurred in 7 patients with original conservative surgery for extremity (5 patients, wide surgical margins) and axial location (2 patients, intralesional surgical margins).

TABLE 1. Patient Characteristics at the Time of Primary Diagnosis and at the Time of First Relapse (n = 31)

Characteristics	n (%)/Median (Range)
Primary diagnosis	
Age (y)	13.7 (1.9-21.0)
Sex	
Male	15 (48)
Female	16 (52)
Site of primary disease	
Femur	19 (62)
Tibia	6 (19)
Humerus	3 (10)
Spine	2 (6)
Shoulder blade	1 (3)
Primary chemotherapy	
CDDP/DOXO/HD-MTX/BCD	9 (29)
CDDP/DOXO/HD-MTX/IFOS	12 (39)
CDDP/DOXO/HD-MTX/IFOS/VP-16	10 (32)
Primary surgery	
Limb sparing	26 (84)
Amputation	5 (16)
Histologic response (%)	50 (0-100)*
First relapse	
Site of disease recurrence	
Lung alone	16 (52)
Isolated local	7 (23)
Lung and distant bone	3 (10)
Lung and local	2 (6)
Local and distant bone	2 (6)
Isolated distant bone	1 (3)
Relapse during treatment	11 (36)
Lung involvement (n = 21)	
Unilateral	8 (38)
Bilateral	13 (62)
No. lung metastases (n = 21)	
1	5 (24)
2	3 (14)
≥3	13 (62)
Treatment	
Chemotherapy + surgery	16 (52)
Chemotherapy	8 (26)
Surgery	5 (16)
None	2 (6)

*Data were only available in 26/31 patients.

BCD indicates bleomycin, cyclophosphamide, actinomycin-D; CDDP, cisplatin; DOXO, doxorubicin; HD-MTX, high-dose methotrexate; IFOS: ifosfamide; VP-16, etoposide.

Treatment After First Relapse

Most patients were treated with chemotherapy in combination with surgery (Table 1). Relapse chemotherapy was mainly based on agents different from those given at initial diagnosis (ifosfamide/cyclophosphamide ± etoposide in 17/24 patients).

Five patients were treated with surgery alone, 3 of whom presented a solitary pulmonary nodule. Two patients treated before the 90s received only palliative care because of relapse during adjuvant first-line chemotherapy resulting in an unresectable tumor.

Outcome After First Relapse

One third of the patients who achieved a CR2 are long-term survivors, as well as 1 patient with a third CR after solitary pulmonary metastasis followed by local relapse (Fig. 1). Median PREFS for patients with CR2 was 22 months (range, 3 to 271 mo).

Two patients with isolated local relapse who underwent radical surgery followed by adjuvant chemotherapy and 4 patients with exclusive lung metastatic relapse with complete surgical resection ± adjuvant chemotherapy are alive in CR. One patient is currently on palliative care. None of the other relapsing patients survived. Disease progression was the cause of death in all of them. PRS and PREFS at 5 years were both 26% (95% confidence interval, 14%-49%), with a median follow-up of 99 months for survivors (range, 27 to 271 mo) after first relapse (Fig. 2).

Prognostic Factors at First Relapse

None of the initial disease presentation/treatment factors correlated with survival in the univariate analysis (age, sex, site of relapse, histologic response). Among relapse presentation and treatment, only time to first relapse and relapse during adjuvant first-line chemotherapy, as well as surgery and achievement of CR2, correlated with improved overall survival ($P < 0.001$, all) (Table 2 and Fig. 3).

In the multivariate analysis, relapse during adjuvant first-line chemotherapy and surgery was not included in the analysis because of overlap with other variables (time to first relapse and achievement of CR2). Achievement of CR2 ($P < 0.001$) and histologic response to first-line treatment ($P = 0.02$) were significantly associated with PRS, whereas time to first relapse did not retain univariate significance (Table 3).

DISCUSSION

Approximately 80% to 90% of localized high-grade osteosarcoma patients achieve a CR to upfront therapy; however, one third of them relapse with poor prognosis. In our study, we found that achievement of CR2 and histologic response to preoperative first-line treatment are independent survival prognostic factors in osteosarcoma recurrence, whereas time to first relapse loses statistical significance when included in a multivariate model.

Patient characteristics in our study at initial diagnosis and at relapse are similar to those previously reported,[5–14] with the exception of local relapse frequency and histologic response to first-line preoperative chemotherapy. In our study, 7 patients (23%) presented with isolated local relapse and 4 patients (13%) with combined local/metastatic relapse. This proportion is similar to the single-institution experience described by Crompton et al[12] (22% and 8%, respectively), but higher than most other publications (5% to 12% and 3% to 8%).[5,8,10,11,16] This is probably because of the few patients with good histologic response to preoperative first-line chemotherapy in our study (4 patients, 15%), a well-known independent prognostic factor for local relapse,[17] compared with the 27% to 56% of good responders previously reported.[5,6,16] In our study, 26 patients

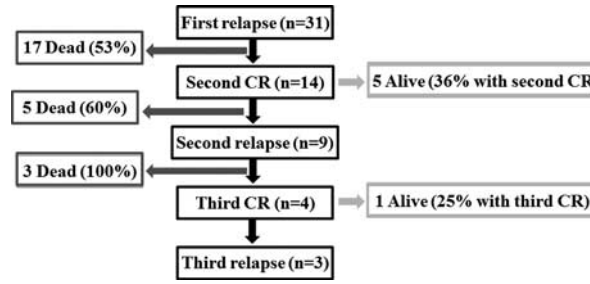

FIGURE 1. Clinical course for all patients (n = 31). CR indicates complete response.

(84%) underwent conservative surgery, raising the concern of its correlation with higher rate of local relapse; however, it is currently clear that only the type of surgical margins and grade of histologic response to preoperative chemotherapy, and not type of surgery, correlate with the risk of local relapse.[17]

In our cohort, 5-year PRS and PREFS were both 26% (95% confidence interval, 14%-49%), with a median follow-up of 99 months (range, 27 to 271 mo) after first relapse, similar to the 10% to 36% previously published.[5–7,9,10,13] Time to first relapse, occurrence of relapse during adjuvant first-line chemotherapy, surgical treatment of relapse, and achievement of CR2 correlated with improved overall survival (all, $P < 0.001$).

Several studies have reported a direct correlation between increased survival after disease recurrence and longer time to first relapse, probably because of less aggressive disease in the late recurrence setting, with different cutoff points considered: 18 months,[5,8] 21 months,[9] or 24 months.[6,7,10,11] In our study, median time to first relapse was significantly longer for survivors (24 vs. 11 mo, $P < 0.001$). None of the 11 patients who relapse during first-line adjuvant chemotherapy survived ($P < 0.001$).

With regard to treatment after relapse, complete surgical resection is essential for outcome, whereas the role of second-line chemotherapy is less clear.[5–14] In our study, 21 patients underwent surgical recurrence resection, 14 achieved a CR2, and 6 of them are long-term survivors ($P < 0.001$). Chemotherapy was usually reserved for patients with unresectable tumors or those with short disease-free interval and/or multiple metastases and did not correlate with survival. Although none of our patients with

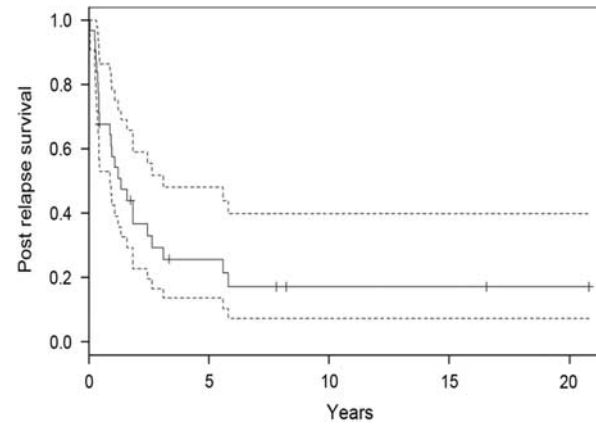

FIGURE 2. Postrelapse survival (95% confidence interval).

TABLE 2. Univariate Analysis of Prognostic Factors

Variables	n (%)/Median (Range) Survivors (n = 7)	Exitus (n = 24)	*P*
Age at diagnosis (y)	11.5 (10.0-21.0)	14.1 (1.9-20.6)	0.73
Sex			
Male	1 (14)	14 (58)	0.12
Female	6 (86)	10 (42)	
Site of relapse			
Lung/bone only	4 (57)	13 (54)	0.32
Local only	2 (29)	5 (21)	
Combined relapse	1 (14)	6 (25)	
Time to first relapse (mo)	24 (16-36)	11 (3-32)	< 0.001
Histologic response (first CR)	70 (20-95)	40 (0-100)	0.10
Relapse during treatment	0	11 (46)	< 0.001
Lung involvement			0.11
Unilateral	3 (75)	5 (29)	
Bilateral	1 (25)	12 (71)	
No. lung metastases			0.23
1	2 (50)	3 (18)	
2	1 (25)	2 (12)	
≥ 3	1 (25)	12 (70)	
Surgery at first relapse	7 (100)	14 (58)	< 0.001
Chemotherapy at first relapse	5 (71)	19 (80)	0.91
Achieved second CR	6 (86)	8 (33)	< 0.001

CR indicates complete response.

third relapse remained alive, survival after multiple relapses is still possible, as shown by a larger series with 5-year PRS and PREFS of 28% and 13%.[18]

The Kaplan-Meier method and the log-rank test are widely used for estimating survival. However, these methods only provide unadjusted survival probabilities. To estimate the true effect of a single variable, it is essential that the results are adjusted for the potential confounding effect of other variables with multivariate comparison methods such as the Cox regression model.[19,20] In the multivariate analysis, time to first relapse ($P = 0.82$) was not an independent survival prognostic factor. As summarized in Table 4, half of the largest studies analyzing prognostic factors in relapse osteosarcoma have not applied multivariate analysis.[8,10–12,14]

Histologic response to preoperative chemotherapy has been classically classified according to the Huvos grading system into 4 types: grade I (0% to 49% necrosis), grade II (50% to 89% necrosis), grade III (90% to 99% necrosis), and grade IV (100% necrosis).[21] According to several studies since Rosen et al,[22] patients with tumor necrosis < 90% are considered as poor responders.[5,6,14] However, as necrosis rate varies widely between 0-89%, we have therefore analyzed histologic response as a continuous variable in our study.

Time to first relapse is a classic prognostic factor in relapse osteosarcoma,[5–11] as well as in other tumors. However, in our small cohort of patients, the worse prognosis observed in early relapsing patients could be explained by histologic response. Hence, those patients with poorer histologic response to preoperative first-line chemotherapy relapsed earlier and did poorer. Both variables were either not assessed in previously published multivariate analysis[6,7,9,13] or not included as continues variables[5] (Table 4). Categorization/dichotomization of continuous variables usually makes interpretation simpler but might lead to considerable loss of power and to biased results.[23,24]

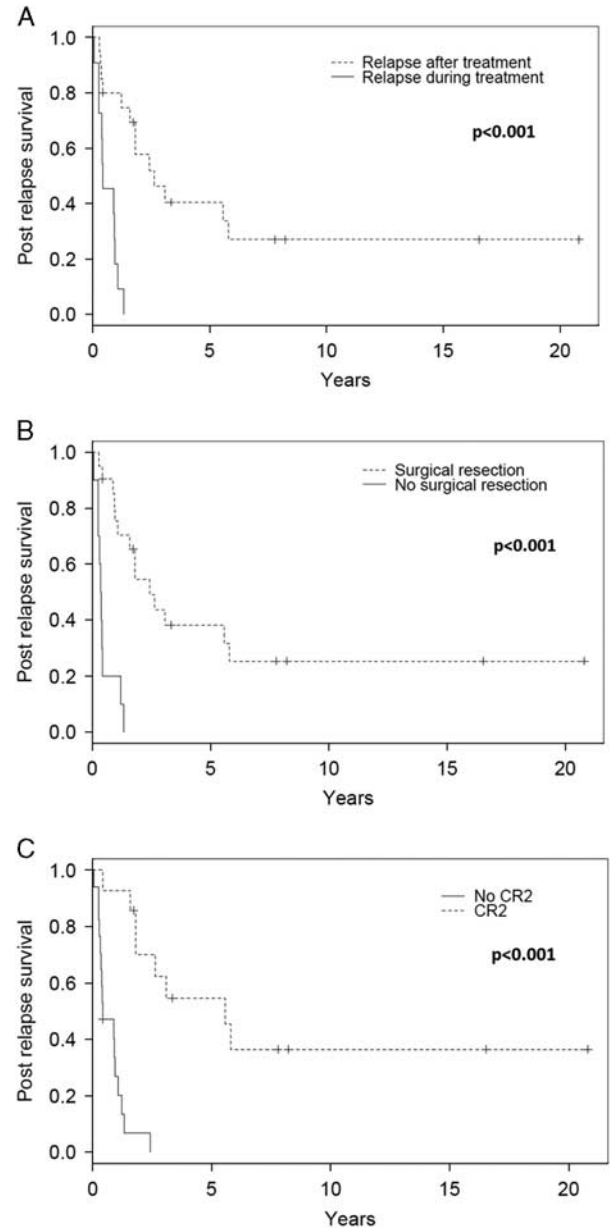

FIGURE 3. Survival after first recurrence. CR at the end of first-line chemotherapy (A), surgery at relapse (B), and achievement of CR2 (C). CR2 indicates second complete response.

TABLE 3. Multivariate Analysis of Prognostic Factors

Variables	Hazard Ratio	95% CI	*P*
Achievement of second CR	0.016	0.002-0.156	< 0.001
Histologic response	0.976	0.956-0.996	0.020
Time to first relapse	1.001	0.934-1.090	0.821

CI indicates confidence interval; CR, complete response.

TABLE 4. Summary of Studies Analyzing Prognostic Factors in Relapse Osteosarcoma After Achievement of First Complete Response

References	n	Period	Age at Diagnosis (y)	Histologic Response	Time to Relapse (y)	CR2 (%)	Follow-up (y)	PRS (95%CI) (%)	Univariate Prognostic Factors	Multivariate Prognostic Factors
Kempf-Bielack et al[5]	576*	1979-1998	15.5 (2.2-68.2)	> 90%: 41.8% ≤10%: 58.2%	1.6 (0.1-14.3)	59	4.2 (0.1-18.4)	5y: 23 (21-26)	Histologic response Time to relapse (> 18 mo) Solitary lesion Pleural integrity Surgery Chemotherapy Radiotherapy	Time to relapse (> 18 mo) Solitary lesion Pleural integrity Surgery Chemotherapy †
Gelderblom et al[6]	564‡	1983-2002	15 (3-40)§	> 90%: 26.7% ≤10%: 73.3%	1.1 (NG-NG)	NG	2.6 (NG-NG)	5y: 18 (15-22)	Time to relapse (≥ 24 mo) Site of first recurrence Histologic response Surgery type	Time to relapse (≥ 24 mo) Site of first recurrence Histologic response Surgery type‖
Ferrari et al[7]	175‡	1986-1995	17.5 (5-47)*	NG	1.9 (0.2-11.4)	70	5.0 (0.1-9.3)	5y: 28 (21-35)	Time to relapse (> 24 mo) Lung metastases (≤2)	Time to relapse (> 24 mo) Lung metastases (≤2)†
Leary et al[8]	110*	1970-2004	14.2 (5.9-25.1)	NG	1.3 (0.1-6.6)	56	13.7 (0.1-33.5)	10y: 17 (13-21)	Time to relapse (≥ 18 mo) Achievement of CR2 Surgery Chemotherapy	ND
Saeter et al[9]	60‡	1975-1993	17 (5-44)§	NG	1.1 (0.1-6.5)		5.7 (0.9-15.8)	5y: 24 (13-36)	Time to relapse (≥ 21 mo) Solitary lesion Achievement of CR2 Chemotherapy	Solitary lesion Achievement of CR2 Chemotherapy
Hawkins and Arndt[10]	59*	1990-2000	15.3 (4.5-23.0)	NG	1.3 (0.2-7.7)	68	4.8 (0.1-10.0)	4y: 23 (10-36)	Time to relapse (> 24 mo) Achievement of CR2 Unilateral lung metastases	ND
Chou et al[11]	43*	1990-2004	15.0 (4.5-31.4)	NG	1.8 (0.4-11.3)	60	1.3 (01-13.2)	3y: 35 (20-50)	Time to relapse (> 24 mo) Achievement of CR2	ND
Tabone et al[14]	42‡	1981-1993	12 (4-18)	> 90%: 40.5% ≤10%: 59.5%	1.8 (0.4-5.0)		3.5 (0.9-11.5)	3y: 36 (NG)	Achievement of CR2	ND
Crompton et al[12]	37‡	1974-1996	16.4 (4.9-30.6)	NG	1.2 (0.2-4.2)	60	NG (6-24)	10y: 15 (NG)	NG	ND
Duffaud et al[13]	33‡	1979-1998	20 (12-55)	NG	1.7 (0.2-12.0)	58	3.3 (0.3-10.3)	3y: 31.6 (NG) 5y: 23.7 (NG)	Achievement of CR2 Surgery	Achievement of CR2
This study	31*	1985-2011	13.7 (1.9-21.0)	> 90%: 15% ≤10%: 85%	1.3 (0.3-3.0)	45	8.2 (0.1-22.6)	5y: 26 (14-49)	Time to relapse Achievement of CR2	Achievement of CR2 Histologic response

Median (range).
*Recurrent OS after CR1.
†CR2 analyzed separately.
‡Recurrent nonmetastatic extremity OS.
§Age at first relapse.
‖CR2 data are not included.
CI indicates confidence interval; CR, complete response; ND, not done; NG, not given; PRS, postrelapse survival.

Our study limitations are the small number of patients and the long inclusion period (27 y) with different chemotherapy regimens, although all patients received high-dose methotrexate, doxorubicin, and cisplatin as first-line chemotherapy treatment. In addition, all essential prognostic factors previously described could be retrospectively reviewed. Nevertheless, histologic response was not evaluable in 5 patients, and therefore multivariate analysis could only be implemented in 26 patients. Another important limitation is that the multivariate model is only adjusted for 3 variables according to the number of patients, and other confounding factors cannot be excluded.

Prospective multicenter data collection in relapsed osteosarcoma and complete statistical analysis of prognostic factors in osteosarcoma recurrence are needed. Considering the extremely poor prognosis of unresectable relapsed osteosarcoma, these patients are excellent candidates for prospective trials that evaluate new therapeutic agents and go deeper in osteosarcoma recurrence biology.

In conclusion, achievement of CR2 and histologic response to preoperative first-line treatment are independent survival prognostic factors in osteosarcoma recurrence. In our cohort, time to first relapse did not retain univariate statistical significance. Prospective multicenter data collection, new therapeutic agent trials, and further study of osteosarcoma biology are needed.

REFERENCES

1. Ries LAG, Smith MA, Gurney JG, Linet M, Tamra T, Young JL, Bunin GR. *Cancer Incidence and Survival Among Children and Adolescents: United States SEER Program 1975-1995*. Bethesda, MD: National Cancer Institute, SEER Program; 1999. NIH Pub. No. 99-4649.
2. Bielack S, Jurgens H, Jundt G, et al. Osteosarcoma: the COSS experience. *Cancer Treat Res*. 2009;152:289–308.
3. Ferrari S, Palmerini E, Staals EL, et al. The treatment of nonmetastatic high grade osteosarcoma of the extremity: review of the Italian Rizzoli experience. Impact on the future. *Cancer Treat Res*. 2009;152:275–287.
4. Jaffe N, Puri A, Gelderblom H. Osteosarcoma: evolution of treatment paradigms. *Sarcoma*. 2013;2013:203531.
5. Kempf-Bielack B, Bielack SS, Jurgens H, et al. Osteosarcoma-relapse after combined modality therapy: an analysis of unselected patients in the Cooperative Osteosarcoma Study Group (COSS). *J Clin Oncol*. 2005;23:559–568.
6. Gelderblom H, Jinks RC, Sydes M, et al. Survival after recurrent osteosarcoma: data from 3 European Osteosarcoma Intergroup (EOI) randomized controlled trials. *Eur J Cancer*. 2011;47:895–902.
7. Ferrari S, Briccoli A, Mercuri M, et al. Postrelapse survival in osteosarcoma of the extremities: prognostic factors for long-term survival. *J Clin Oncol*. 2003;21:710–715.
8. Leary SE, Wozniak AW, Billups CA, et al. Survival of pediatric patients after-relapsed osteosarcoma: the St Jude Children's Research Hospital experience. *Cancer*. 2013;119:2645–2653.
9. Saeter G, Hoie J, Stenwig AE, et al. Systemic-relapse of patients with osteogenic sarcoma. Prognostic factors for long term survival. *Cancer*. 1995;75:1084–1093.
10. Hawkins DS, Arndt CA. Pattern of disease recurrence and prognostic factors in patients with osteosarcoma treated with contemporary chemotherapy. *Cancer*. 2003;98:2447–2456.
11. Chou AJ, Merola PR, Wexler LH, et al. Treatment of osteosarcoma at first recurrence after contemporary therapy: the Memorial Sloan-Kettering Cancer Center experience. *Cancer*. 2005;104:2214–2221.
12. Crompton BD, Goldsby RE, Weinberg VK, et al. Survival after recurrence of osteosarcoma: a 20-year experience at a single institution. *Pediatr Blood Cancer*. 2006;47:255–259.
13. Duffaud F, Digue L, Mercier C, et al. Recurrences following primary osteosarcoma in adolescents and adults previously treated with chemotherapy. *Eur J Cancer*. 2003;39:2050–2057.
14. Tabone MD, Kalifa C, Rodary C, et al. Osteosarcoma recurrences in pediatric patients previously treated with intensive chemotherapy. *J Clin Oncol*. 1994;12:2614–2620.
15. Berlanga P, Cañete A, Diaz R, et al. Presentation and long-term outcome of high-grade osteosarcoma: a single-institution experience. *J Pediatr Hematol Oncol*. 2015;37:272–277.
16. Bacci G, Briccoli A, Longhi A, et al. Treatment and outcome of recurrent osteosarcoma: experience at Rizzoli in 235 patients initially treated with neoadjuvant chemotherapy. *Acta Oncol*. 2005;44:748–755.
17. Bacci G, Forni C, Longhi A, et al. Local recurrence and local control of non-metastatic osteosarcoma of the extremities: a 27-year experience in a single institution. *J Surg Oncol*. 2007;96:118–123.
18. Bielack SS, Kempf-Bielack B, Branscheid D, et al. Second and subsequent recurrences of osteosarcoma: presentation, treatment, and outcomes of 249 consecutive cooperative osteosarcoma study group patients. *J Clin Oncol*. 2009;27:557–565.
19. Stel VS, Dekker FW, Tripepi G, et al. Survival analysis I: the Kaplan-Meier method. *Nephron Clin Pract*. 2011;119:83–88.
20. Stel VS, Dekker FW, Tripepi G, et al. Survival analysis II: Cox regression. *Nephron Clin Pract*. 2011;119:255–260.
21. Huvos AG, Rosen G, Marcove RC. Primary osteogenic sarcoma: pathologic aspects in 20 patients after treatment with chemotherapy en bloc resection, and prosthetic bone replacement. *Arch Pathol Lab Med*. 1977;101:14–18.
22. Rosen G, Caparros B, Huvos AG, et al. Preoperative chemotherapy for osteogenic sarcoma: selection of postoperative adjuvant chemotherapy based on the response of the primary tumor to preoperative chemotherapy. *Cancer*. 1982;49:1221–1230.
23. Royston P, Altman DG, Sauerbrei W. Dichotomizing continuous predictors in multiple regression: a bad idea. *Stat Med*. 2006;25:127–141.
24. van Walraven C, Hart RG. Leave'em alone—why continuous variables should be analyzed as such. *Neuroepidemiology*. 2008;30:138–139.

ARTÍCULO III

Cancer in cancer in children and adolescents in Spain: incidence, treatment setting and provider specialty.

Pablo Berlanga, María Luisa Vicente, Adela Cañete, Carmen Alberich, Victoria Castel.

Clin Transl Oncol 2016; 18: 27-32.
Impact Factor 2015: 2.075

Clin Transl Oncol (2016) 18:27–32
DOI 10.1007/s12094-015-1330-y

RESEARCH ARTICLE

Cancer in children and adolescents in Spain: incidence, treatment setting and provider specialty

P. Berlanga[1,3] · M. L. Vicente[2] · A. Cañete[1,3] · C. Alberich[2] · V. Castel[3]

Received: 27 February 2015 / Accepted: 13 June 2015 / Published online: 25 June 2015

Abstract
Purpose To analyze cancer incidence, distribution of malignancy, treatment setting and provider specialty of cancer patients, 0–19 years old, in the Comunitat Valenciana, Spain.
Methods/patients All incident childhood and adolescent (0–19 years) cancer cases registered in the population-based Comunitat Valenciana Childhood Cancer Registry (RTICV) from 2007 to 2010 were included. Pathological and hematological diagnoses were recoded using the International Classification of Childhood Cancer Third Edition (ICCC-3). Treatment setting and provider specialty were analyzed.
Results 696 patients <20 years were diagnosed with cancer: 513 cases were children (0–14 years) and 183 were adolescents (15–19 years). Overall age-adjusted incidence for 2007–2010 was 176.0 cases per million (95 % CI 162.8–189.2), with incidence being the highest among infants (287.4), followed by 1–4 years (205.5), adolescents (179.9), 10–14 years (150.2) and 5–9 years (140.6). Among adolescents aged 14–19 years, the treatment setting differed by cancer type; 87 % of them were never seen at pediatric oncology units, while 40 % were treated in up to 20 different medical oncology departments in institutions without pediatric oncology expertise.
Conclusions This is the first population-based epidemiological study carried out in Spain on children and adolescents with cancer. Centralization of care to a small number of specialized centers and thorough pediatric and oncology team collaboration are needed to improve care and survival for adolescents with cancer in our country. We suggest the creation of specific adolescent tumor boards in main tertiary care hospitals, in which adolescents with cancer can benefit from the shared expertise of medical and pediatric specialists.

Keywords Population-based cancer registries · Cancer incidence · Treatment setting · Children · Adolescent · Spain

✉ P. Berlanga
berlanga_pab@gva.es

[1] Pediatric Oncology Unit, Hospital La Fe, Avda Fernando Abril Martorell, no. 106, 46026 Valencia, Spain

[2] Public Health Directorate General, Valencian Government Health Department, Valencia, Spain

[3] Cancer Clinical and Translational Research Group, Health Research Institute La Fe, Valencia, Spain

Introduction

Adolescents are a unique age group, with patterns of disease and healthcare challenges distinctly different than those faced by younger children and adults. Teenagers from 15 to 19 do not only have a higher overall incidence of malignancy than children, but also a different disease distribution to children and adults [1–4]. Unlike younger or older patients, adolescents may be referred by pediatricians/general physicians to either pediatric or adult oncologists. A definitive association between treatment setting and outcome in this group of patients has not yet been established. However, recent data suggest that adolescents with cancer are not treated at optimal settings, enrolled in clinical trials at low rates and, for certain tumor types, outcome may be better when care is delivered in a pediatric center or according to a pediatric protocol [5–7].

Approximately 400–450 adolescents aged 15–19 years are estimated to be diagnosed with cancer each year in

Spain based on the incidence rate in Europe and the 15–19 years old population in Spain [3, 8, 9]; however, no real incidence data are available in our country. Most patients treated in Spanish pediatric oncology centers are usually registered in the Spanish Childhood Cancer Registry (RETI-SEHOP). The RETI-SEHOP is a hospital-based cancer registry that collects cases from all Spanish pediatric oncology centers and covers the entire country, with an estimated average completeness of 90 % in the 0–14 years old group [10]. However, concerning the 15–19 years old group, there is an important lack of information regarding cancer incidence, pattern of referral, treatment setting and provider specialty. Spain is divided in 17 regions (Autonomous Communities) and the pediatric age cutoff ranges from 14 to 18 years old not just among different regions but also among different hospitals within the same community.

Comunitat Valenciana is one of the seventeen regions in Spain. It is located in the east coast and covers a total population of 5.1 million, including ca. 1 million children and adolescents which represents 11 % of the 0–19 years old population in Spain [9] (Fig. 1). The Comunitat Valenciana Childhood Cancer Registry (RTICV) is the only population-based childhood & teenager cancer registry in Spain. It depends on the Public Health Directorate General, Health Department of Valencian Government and collects childhood cancer data (0–14 years) since 1983, as well as adolescent data (15–19 years) since 2007 covering 100 % of the Comunitat Valenciana population.

Our objective is to determine the incidence, distribution of malignancy, treatment setting and provider specialty of cancer patients 0–19 years old in the Comunitat Valenciana, with the aim to estimate adolescent cancer incidence in our region and to compare their demographic, disease and clinical characteristics according to treatment setting and provider specialty.

Methods

For the purpose of this study, all incident childhood and adolescent (0–19 years) cancer cases registered in the Comunitat Valenciana Childhood Cancer Registry (RTICV) from 2007 to 2010 were included. Residence status was determined through insurance card and, if available, identity card, as well as confirmed through systematic review of medical records. Non-resident cancer cases at the time of diagnosis were excluded.

Individual pathological and hematological diagnoses were reviewed and recoded using the International Classification of Childhood Cancer Third Edition (ICCC-3) into 12 main diagnosis groups [11]. RTICV usually identifies incident cases of cancer through routine and systematic review of pathology reports, medical records, radiation therapy records and hospital discharge list from the 21 public and 5 private hospitals in our region. Estimate of completeness of case coverage was of 98 % during the study period. Annually, information of cases treated in all hospitals of the CV is requested, and identified patients are compared with the database generated with cases from previous years. The information on possible cases is verified and completed by consulting medical history, and contrasted with the demographics of the Population Information System and Mortality Registry. Indicators of data quality of the Registry in this period were: 94.7 % microscopic verification (MV), 0 % Death Certificate Only (DCO) registrations; and 0.03 % cases with unspecified histology in subgroups and 0 % with unspecified histology in group diagnostic.

Treatment setting and provider specialty were also retrospectively analyzed. Data were provided anonymized by the RTICV and therefore considered exempt of the ethics panel.

Standard variables for each case included demographic data (birth date, age, sex, residence at diagnosis) and

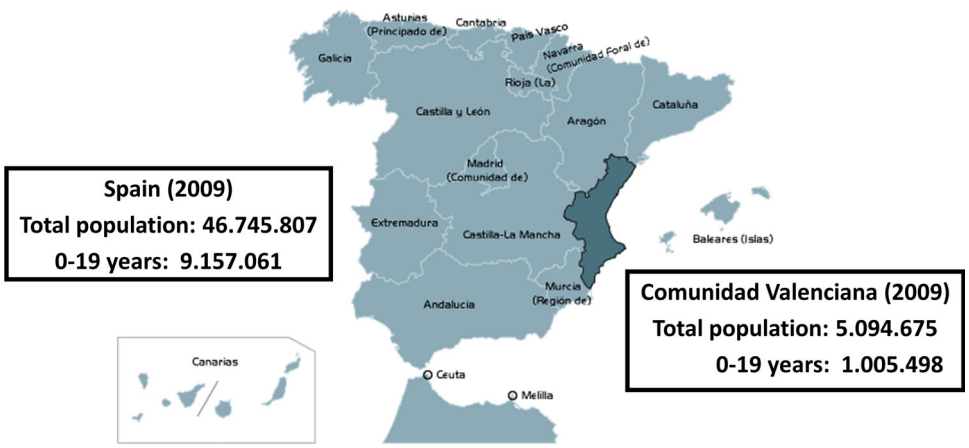

Fig. 1 Spain and the Comunitat Valenciana, National Statistics Institute (2007–2010)

clinical data (diagnosis date, ICCC-3 diagnostic group), provider specialty (pediatric vs medical oncologist) and treatment setting. The population at risk in the period was 3.964.716 person-years, obtained from the National Statistics Institute [9]. Based on the pediatric age cutoff in our region, patients were grouped in two (<14 and ≥14 years) for treatment setting and provider specialty analysis. For incidence and distribution of malignancy analysis, the usual age grouping was employed (0, 1–4, 5–9, 10–14 and 15–19 years). In our region there are 21 public and 5 private medical oncology services, as well as three public pediatric oncology units. All three pediatric oncology units have pediatric oncologists/surgeons on staff and are located within a tertiary care hospital with full access to appropriate pediatric subspecialties and pediatric supportive care. For this analysis, different centers of treatment were aggregated into the following groups: (1) Pediatric Oncology Center, (2) Medical Oncology Center with a pediatric oncology center within the same institution, (3) Medical Oncology Center without a pediatric oncology center within the same institution.

Statistical analysis

Incidence rates were calculated by specific age groups and crude rate for all groups by sex and adjusted standardized rates by world population. Categorical variables were described with the numerical count (percentage) of each category and 95 % confidence intervals (CI). Categorical variables were compared using Chi-squared test. $p < 0.05$ was considered as statistically significant. Data were analyzed using SPSS 20.0 and Epidat 3.1.

Results

From January 2007 to December 2010, there were 696 patients younger than 20 years, residents in the Comunitat Valenciana and diagnosed with a new cancer. Five hundred and thirteen cases were children between 0 and 14 years old and 183 were adolescents between 15 and 19 years. Diagnosis was microscopically verified in 94.7 % of cases.

Overall age-adjusted incidence for 2007–2010 was 176.0 cases per million (95 % CI 162.8–189.2), with incidence being the highest among infants (287.4), followed by 1–4 years (205.5), adolescents (179.9), 10–14 years (150.2) and 5–9 years (140.6). In children (0–14 years) leukemia was the most common diagnosis, followed by CNS tumors, lymphomas, bone tumors and neuroblastoma. In adolescents (15–19 years), lymphoma was the most common diagnosis, followed by CNS tumors, leukemia, other carcinomas/melanomas and bone and germ cell tumors (Table 1).

A total of 499 patients were treated at pediatric oncology units (72 %; 95 % CI 68–75 %). The proportion of patients treated at a pediatric oncology center declined with age (Fig. 2). Nearly all children younger than 14 years old with cancer received their oncology care at a pediatric oncology unit, while only 57 % (95 % CI 39–75 %) of 14-year-olds and 4 % (95 % CI 1–7 %) of 15–19-year-olds ($p = 0.001$) (Table 2), Accordingly, 14 years of age has been considered the cutoff point for the analysis of treatment setting and provider specialty in our cohort.

Only 13 % (95 % CI 8–17 %) of ≥14 years old were treated in Pediatric Oncology Units; 47 % (95 % CI 40–54 %) were treated at Medical Oncology Departments with a pediatric oncology unit within the same institution whereas 40 % (95 % CI 34–47 %) at a Medical Oncology Department without.

Regarding different centers of treatment 60 % (95 % CI 53–67 %) of 14–19-year-olds were treated in one of the three main tertiary care hospitals of the Comunitat Valenciana (whether in the pediatric or medical oncology center), while 40 % (95 % CI 34–47 %) were treated in one of the remaining 20 different medical oncology departments (median 4 patients/4 years period, range 1–15 patients). The percentage of 14–19-year-olds treated in one of the three main tertiary care hospitals in the Comunitat Valenciana increased from 56 % (95 % CI 42–69 %) in 2007 up to 72 % (95 % CI 59–85 %) in 2010 ($p = 0.17$). Among adolescents aged 14–19 years, the treatment setting differed by diagnostic (Table 2). When compared by diagnostic category, malignancies such as leukemia and bone and soft tissue sarcomas were more frequently treated in one of the three main tertiary care hospitals (whether in the pediatric or medical oncology center), than malignancies such as germ cell tumors and other carcinomas/melanomas ($p = 0.001$),

Discussion

This is the first population-based epidemiological study carried out in Spain on adolescents with cancer. The data presented come from the only population-based specialized childhood and teenager cancer registry in Spain, RTICV, which covers 100 % of the 0–19-year-olds of the Comunitat Valenciana population [12]. Population-based cancer registries, such as the RTICV, record all new cases in a geographically defined area, independently of provider specialty, and are thus unique for monitoring cancer incidence and provide indicators for planning and evaluating cancer control activities [12, 13].

In our study, 513 patients were ≤14 years at cancer diagnosis and 183 were between 15 and 19 years. Childhood and adolescent cancer incidence is similar to

Table 1 Cancer incidence age-standardized rate (ASRw) per million person-years in the Comunitat Valenciana (2007–2010) by age groups according to the ICCC-3

Diagnostic group (ICCC-3)	0 years old	1–4 years old	5–9 years old	10–14 years old	15–19 years old	ASRw (95 % CI)
I. Leukemias	55.5	76.0	51.3	22.4	27.7	45.7 (38.9–52.5)
II. Lymphomas	10.1	16.6	16.4	29.8	45.5	25.6 (20.7–30.5)
III. CNS neoplasms	40.3	36.8	39.0	33.0	33.6	36.0 (30.1–42.0)
IV. Neuroblastoma	70.6	28.5	2.1	1.1	1.0	12.1 (8.4–15.7)
V. Retinoblastoma	15.1	7.1	1.0	0.0	0.0	2.9 (1.1–4.7)
VI. Renal tumors	20.2	17.8	4.1	1.1	0.0	6.8 (4.0–9.5)
VII. Liver tumors	20.2	3.6	0.0	1.1	1.0	2.5 (0.9–4.2)
VIII. Bone tumors	0.0	3.6	11.3	30.9	19.8	15.1 (11.3–18.8)
IX. Soft tissue sarcoma	25.2	10.7	10.3	7.5	8.9	10.3 (7.1–13.5)
X. Germ cell tumors	15.1	3.6	1.0	9.6	16.8	8.0 (5.2–10.7)
XI. Other carcinomas/melanomas	15.1	1.2	4.1	13.8	25.7	11.1 (7.9–14.3)
Total	287.4	205.5	140.6	150.2	179.9	176.0 (162.8–189.2)

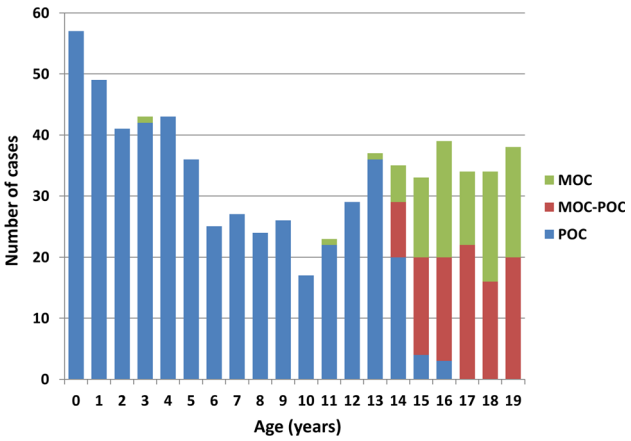

Fig. 2 Site of care for cancer patients in the Comunitat Valenciana by age (2007–2010). *POC* Pediatric Oncology Center, *MOC-POC* Medical Oncology Center with a Pediatric Oncology Center within the same institution, *MOC* Medical Oncology Center without a Pediatric Oncology Center within the same institution

previously described with higher overall incidence of malignancy in adolescents than in children. [3, 14, 15]. Nearly all children younger than 14 years old with cancer received their oncology care at a pediatric oncology unit, whereas only 4 % of 15–19-year-olds was treated at pediatric units compared to 30–47 % in other countries [16–19]. This decline in utilization of pediatric units is explained by the current pediatric age cutoff (14 years old) in the Comunitat Valenciana as well as in most Spanish regions.

Cancer in adolescents represents a transition between the non-epithelial types common during childhood and the epithelial types that account for most cancers in adults [1–4, 14]. Compared to younger or older patients, adolescents may be referred to pediatric or adult oncologists depending on referral physician and/or center of treatment policy. This unique pattern of disease and pattern of referral means that few institutions develop the required expertise, infrastructure or clinical trial opportunities for better management of the adolescents with cancer.

In our study, while 60 % of 14–19-year-olds were treated in the three main tertiary care hospitals (within the pediatric or medical center), 40 % were treated in one of the remaining 20 different medical oncology departments. For this study, only the main treatment center was recorded and therefore the number of treating centers per patient cannot be presented. The type of cancer had a strong influence on site of care: those more common during childhood such as leukemia and bone/soft tissue sarcomas were mostly centralized in one of the three main tertiary care hospitals, whereas those more common during adulthood such as germ cell tumors and other carcinomas/melanomas were independently referred to medical oncology departments. Multiple treatment centers and lack of pediatric cancer expertise within the same institution (not only oncologists and surgeons, but pathologists, radiologists, etc.) may hamper access to best cancer diagnosis and treatment for this group of patients.

Different international consensuses emphasize the importance of specialized care and clearly state that "all care for children and young people under 19 years old must be provided in age-appropriate facilities", "must have access to tumor-specific or treatment-specific clinical expertise", "be offered entry to any clinical research trial for which they are eligible" and "treated according to the best available treatment protocol" [20, 21]. In order to achieve these aims, centralization of care of adolescents with cancer, as already successfully achieved for children,

Table 2 Site of care for 14–19-year-olds with cancer in the Comunitat Valenciana (2007–2010) by diagnosis

	14 years				15–19 years			
	POC	MOC-POC	MOC	Total	POC	MOC-POC	MOC	Total
I. Leukemias	2	0	0	2	5	21	2	28
II. Lymphomas	6	0	1	7	0	22	25	47
III. CNS Neoplasms	4	3	2	9	0	20	14	34
IV. Neuroblastoma	0	0	0	0	0	1	0	1
V. Retinoblastoma	0	0	0	0	0	0	0	0
VI. Renal tumors	0	0	0	0	0	0	0	0
VII. Liver tumors	1	0	0	1	0	1	0	1
VIII. Bone tumors	3	0	3	6	1	16	3	20
IX. Soft tissue sarcoma	2	0	1	3	1	3	5	9
X. Germ cell tumors	2	0	0	2	0	4	13	17
XI. Other carcinomas/melanomas	0	3	2	5	0	8	18	26
Total	20	6	9	35	7	96	80	183

POC Pediatric Oncology Center, *MOC-POC* Medical Oncology Center with a Pediatric Oncology Center within the same institution, *MOC* Medical Oncology Center without a Pediatric Oncology Center within the same institution

will surely improve their care and outcome. This could be achieved through specific teenager and young adult (TYA) cancer centers or integration into current pediatric and/or medical oncology departments. It is worth noting, that in our study, the proportion of adolescents treated at one the three main tertiary care hospitals has clearly been increasing (56 % in 2007, 72 % in 2010), although statistically not significant.

Adolescents treated in pediatric oncology centers are more likely to be enrolled in clinical trials than those who receive their care elsewhere. The lower rate of trial enrollment in this age group is probably responsible, at least in part, of the insufficient improvement in survival, morbidity and quality of life among adolescents compared with children or adults [7]. As most adult clinical trials usually have a lower age limit of 18 years [22], what happens with 14–17-year-olds treated at medical oncology institutions? Collaboration between pediatric and adult oncology teams throughout specific adolescent programs can improve the sharing of expertise and clinical trial enrollment, as shown by the AYA Oncology Program in Pittsburg [23].

In Spain, a new National Strategic Plan for Childhood and Adolescence [24] has recently recommended the extension of current pediatric upper age cutoff to 18 years, facilitating treatment of adolescents in fewer, pediatric units. Nevertheless, current decentralized health care system in our country will hinder the accomplishment of this Plan and advances will have to be achieved at the regional level.

Our study has several limitations. On one hand, due to the usual delay of population-based cancer registries, the last year analyzed in our study is 2010 and may no longer reflect the current situation. RTICV is a population-based registry that needs information of cases treated in all hospitals of the CV in order to verify the cases. However, some hospitals do not provide their data in a timely manner. Nevertheless, as the only new regulatory modification introduced (National Strategic Plan for Childhood and Adolescent [24]) is still not implemented in our region and the pediatric age cutoff has therefore not changed, the data presented still reflect the real situation in our region. Another limitation is that although provider specialty and treatment setting were analyzed, actual degree of collaboration between pediatric and adult specialists could not be retrospectively assessed: some medical oncology departments without pediatric oncology expertise may have consulted with pediatric specialists, while some pediatric and adult specialists within the same institution may have never discussed their adolescent patients. Neither time to diagnosis nor care pathways could be retrospectively assessed. Although distance to cancer treatment was not analyzed in this study, we do not think that the distance between the patient's home and the nearest pediatric oncology center plays a major role, since in all cases they can be reached in less than 2 h by car.

In summary, we have found that most 14–19 years old with cancer in the Comunitat Valenciana in the 2007–2010 period were not seen at pediatric oncology units and almost half of them were treated in institutions without pediatric oncology expertise. Centralization of care to a small number of specialized centers and thorough pediatric and oncology team collaboration are needed to improve care and survival for adolescents with cancer. We suggest the creation of specific adolescent tumor boards in main tertiary care hospitals, in which adolescents with cancer can

benefit from the shared expertise of medical and pediatric specialists.

Acknowledgments This work was supported by the Instituto de Investigación Sanitaria La Fe (2011/0319).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

Informed consent Data were provided anonymized by the Comunitat Valenciana Childhood Cancer Registry (RTICV) and therefore considered exempt of the ethics panel. The RTICV belongs to the Public Health Directorate General, Health Department of Valencian Government. For this type of study, formal consent is not required.

References

1. Bleyer A. Adolescent and young adult (AYA) oncology: the first A. Pediatr Hematol Oncol. 2007;24:325–36.
2. Gatta G, Zigon G, Capocaccia R, Coebergh JW, Desandes E, Kaatsch P, et al. Survival of European children and young adults with cancer diagnosed 1995–2002. Eur J Cancer. 2009;45:992–1005.
3. Stiller CA, Desandes E, Danon SE, Izarzugaza I, Ratiu A, Vassileva-Valerianova Z, et al. Cancer incidence and survival in European adolescents (1978–1997). Report from the Automated Childhood Cancer Information System project. Eur J Cancer. 2006;42:2006–18.
4. Barr RD. Adolescents, young adults, and cancer–the international challenge. Cancer. 2011;117:2245–9.
5. Stock W, La M, Sanford B, Bloomfield CD, Vardiman JW, Gaynon P, et al. What determines the outcomes for adolescents and young adults with acute lymphoblastic leukemia treated on cooperative group protocols? A comparison of Children's Cancer Group and Cancer and Leukemia Group B studies. Blood. 2008;112:1646–54.
6. Paulussen MAS, Juergens HF. Cure rates in Ewing tumor patients aged over 15 years are better in pediatric oncology units: results of GPOH CESS/EICESS studies [abstract]. Proc Am Soc Clin Oncol. 2003;22(816):3279.
7. Tai E, Buchanan N, Westervelt L, Elimam D, Lawvere S. Treatment setting, clinical trial enrollment, and subsequent outcomes among adolescents with cancer: a literature review. Pediatrics. 2014;133(Suppl 3):S91–7.
8. Lassaletta A, Andion M, Garrido-Colino C, Gutierrez-Carrasco I, Echebarria-Barona A, Almazán F, et al. The current situation of adolescents with cancer in pediatric hematology-oncology units in Spain. Results of a national survey. An Pediatr (Barc). 2013; 78:268 e1–7.
9. Instituto Nacional de Estadística. Madrid: INE. http://www.ine.es/inebmenu/mnu_cifraspob.htm. Accessed 11 May 2015.
10. Peris Bonet R, Fuentes García S. Cáncer infantil en España. Estadísticas 1980–2012. Registro Nacional de Tumores Infantiles (RNTI-SEHOP). Valencia: Universitat de València; 2013 (prelimilary issue in CD-ROM). http://www.uv.es/rnti. Accessed 11 May 2015.
11. Steliarova-Foucher E, Stiller C, Lacour B, Kaatsch P. International Classification of Childhood Cancer, third edition. Cancer. 2005;103:1457–67.
12. Navarro C, Martos C, Ardanaz E, Galceran J, Izarzugaza I, Peris-Bonet R, et al: Population-based cancer registries in Spain and their role in cancer control. Ann Oncol 21 Suppl 3:iii3–13, 2010.
13. Parkin DM. The role of cancer registries in cancer control. Int J Clin Oncol. 2008;13:102–11.
14. Ward E, DeSantis C, Robbins A, Kohler B, Jemal A. Childhood and adolescent cancer statistics, 2014. CA Cancer J Clin. 2014;64:83–103.
15. Barr RD, Greenberg ML. Cancer surveillance and control in adolescents–similarities and contrasts between Canada and the United States. Pediatr Blood Cancer. 2006;46:273–7.
16. Albritton KH, Wiggins CH, Nelson HE, Weeks JC. Site of oncologic specialty care for older adolescents in Utah. J Clin Oncol. 2007;25:4616–21.
17. Howell DL, Ward KC, Austin HD, Young JL, Woods WG. Access to pediatric cancer care by age, race, and diagnosis, and outcomes of cancer treatment in pediatric and adolescent patients in the state of Georgia. J Clin Oncol. 2007;25:4610–5.
18. Yeager ND, Hoshaw-Woodard S, Ruymann FB, Termuhlen A. Patterns of care among adolescents with malignancy in Ohio. J Pediatr Hematol Oncol. 2006;28:17–22.
19. Klein-Geltink J, Shaw AK, Morrison HI, Barr RD, Greenberg ML. Use of paediatric versus adult oncology treatment centres by adolescents 15-19 years old: the Canadian Childhood Cancer Surveillance and Control Program. Eur J Cancer. 2005;41:404–10.
20. Kowalczyk JR, Samardakiewicz M, Fitzgerald E, Essiaf S, Ladenstein R, Vassal G. Towards reducing inequalities: European Standards of Care for Children with Cancer. Eur J Cancer. 2014;50:481–5.
21. NICE. Guidance on cancer services—improving outcomes in children and young people with cancer. London: National Institute for Health and Clinical Excellence; 2005.
22. Fern LA, Lewandowski JA, Coxon KM, Whelan J. Available, accessible, aware, appropriate, and acceptable: a strategy to improve participation of teenagers and young adults in cancer trials. Lancet Oncol. 2014;15:e341–50.
23. Shaw PH, Boyiadzis M, Tawbi H, Welsh A, Kemerer A, Davidson NE, et al. Improved clinical trial enrollment in adolescent and young adult (AYA) oncology patients after the establishment of an AYA oncology program uniting pediatric and medical oncology divisions. Cancer. 2012;118:3614–7.
24. Ministerio de Sanidad SSeI: II Plan Estratégico Nacional de Infancia y Adolescencia 2013–2016 (II PENIA). Madrid: Ministerio de Sanidad, Servicios Sociales e Igualdad; 2013.

ARTÍCULO IV

miR-200c and Akt predict osteosarcoma progression and lung metastasis

Pablo Berlanga, Lisandra Muñoz, Marta Piqueras, Antoni Sirerol, María Dolors Sánchez-Izquierdo, David Hervás, Miguel Hernández, Margarita Llavador, Isidro Machado, Antonio Llombart-Bosch, Adela Cañete, Victoria Castel, Jaime Font de Mora.

Mol Oncol. 2016; 10: 1043-53.
Impact Factor 2015: 5.367

MOLECULAR ONCOLOGY 10 (2016) 1043–1053

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/molonc

miR-200c and phospho-AKT as prognostic factors and mediators of osteosarcoma progression and lung metastasis

Pablo Berlanga[a,b], Lisandra Muñoz[h], Marta Piqueras[h], J. Antoni Sirerol[h], María Dolors Sánchez-Izquierdo[c], David Hervás[d], Miguel Hernández[e], Margarita Llavador[e], Isidro Machado[f], Antonio Llombart-Bosch[g], Adela Cañete[a,b], Victoria Castel[a,b], Jaime Font de Mora[b,h,*]

[a]Pediatric Oncology Unit, Hospital Universitario y Politécnico La Fe, Spain
[b]Clinical and Translational Research in Cancer, Instituto de Investigación Sanitaria La Fe, Spain
[c]Genomics Unit, Instituto de Investigación Sanitaria La Fe, Spain
[d]Biostatistics Unit, Instituto de Investigación Sanitaria La Fe, Spain
[e]Department of Pathology, Hospital La Fe, Spain
[f]Department of Pathology, Instituto Valenciano de Oncología, Spain
[g]Department of Pathology, University of Valencia, Spain
[h]Laboratory of Cellular and Molecular Biology, Instituto de Investigación Sanitaria La Fe, Spain

ARTICLE INFO

Article history:
Received 3 February 2016
Received in revised form
23 March 2016
Accepted 15 April 2016
Available online 23 April 2016

Keywords:
Pediatric osteosarcoma
miR-200c
Mesenchymal to epithelial transition
Phospho-AKT
Lung metastasis

ABSTRACT

Lung metastasis is the major cause of death in osteosarcoma patients. However, molecular mechanisms underlying this metastasis remain poorly understood. To identify key molecules related with pulmonary metastasis of pediatric osteosarcomas, we analyzed high-throughput miRNA expression in a cohort of 11 primary tumors and 15 lung metastases. Results were further validated with an independent cohort of 10 primary tumors and 6 metastases. In parallel, we performed immunohistochemical analysis of activated signaling pathways in 36 primary osteosarcomas. Only phospho-AKT associated with lower overall survival in primary tumors, supporting its role in osteosarcoma progression. CTNNB1 expression also associated with lower overall survival but was not strong enough to be considered an independent variable. Interestingly, miR-200c was overexpressed in lung metastases, implicating an inhibitory feed-back loop to PI3K-AKT. Moreover, transfection of miR200c-mimic in U2-OS cells reduced phospho-AKT levels but increased cellular migration and proliferation. Notably, miR-200c expression strongly correlated with miR-141 and with the osteogenic inhibitor miR-375, all implicated in epithelial to mesenchymal transition. These findings contrast epithelial tumors where reduced miR-200c expression promotes metastasis. Indeed, we noted that osteosarcoma cells in the lung also expressed the epithelial marker CDH1, revealing a change in their mesenchymal phenotype. We propose that miR-200c upregulation occurs late in osteosarcoma progression to provide cells with an epithelial phenotype that facilitates their integration in the metastatic lung niche. Thus, our findings identify phospho-AKT in the primary tumor and miR-200c later during

* *Corresponding author*. Fundación para la Investigación Hospital La Fe, Torre A, 5-20, Avda, Fernando Abril Martorell, n°106, 46026, Valencia, Spain. Tel.: +34 961246646; fax: +34 961246232.
E-mail address: jaime.fontdemora@gmail.com (J. Font de Mora).
http://dx.doi.org/10.1016/j.molonc.2016.04.004

tumor progression as prognostic molecules and potential therapeutic targets to prevent progression and metastasis of pediatric osteosarcomas.

1. Introduction

Osteosarcoma is the most common primary high grade bone malignancy with highest incidence in children and adolescents (Arndt et al., 2012). Current survival rates vary by age, disease stage, anatomic site and histologic response to presurgical chemotherapy, ranging from 55 to 75% for children and adolescents in most countries (Savage and Mirabello, 2011). However, survival rate is reduced to 10–30% if there is metastatic disease at diagnosis (Kager et al., 2003). After recurrence, mainly to the lungs, there are limited treatment options that are frequently unsuccessful. Thus, there is an urgent need to identify new druggable targets for precision medicine treatments.

MicroRNAs (miRNAs) are important epigenetic regulators in cellular physiology as well as in the pathogenesis of human cancers, including osteosarcomas. Several miRNAs have already been reported to regulate signaling networks in osteosarcoma cells (reviewed in Chang et al., 2015). Among them, those targeting the insulin-like growth factor-1 IGF-I signaling pathway are of particular interest since this pathway regulates pleiotropic responses that vary from cellular metabolism, proliferation, differentiation and apoptosis. Regulation of the IGF-I signaling pathway and mutations in PI3K, one of its downstream targets, have been described to be of relevance in osteosarcoma, correlating with poor prognosis (Choy et al., 2012; Su et al., 2011). In addition, the monoclonal antibody R1507 targeting IGF-IR was reported to be effective alone or in combination with rapamycin in inhibiting growth of osteosarcoma xenografts (Kolb et al., 2010). Moreover, increased expression of IGF-IR has been correlated with tumor metastasis and prognosis in patients with osteosarcoma (Wang et al., 2012). Notably, an orthotopic study in mice with phospho-specific antibodies and kinase inhibitors revealed that only IGF-IR/MEK pathway, but not IGF-IR/AKT pathway, remain active in lung metastasis, suggesting that Ras/Raf/MEK/ERK signaling may play an important role in osteosarcoma lung metastasis (Yu et al., 2011). In addition, c-Myc overexpression enhanced MG-63 and SAOS-2 osteosarcoma cells invasion through the activation of MEK-ERK pathway whereas inhibited the activity of PI3K-AKT pathway (Han et al., 2012). Further downstream in IGF-I signaling is the Nuclear Receptor Coactivator 3 (NCOA3), which was reported to be overexpressed in osteosarcomas driving its progression (Geng et al., 2014). Strikingly, activation of PI3K/AKT upregulates NCOA3 levels by inhibiting its proteasomal degradation (Ferrero et al., 2008), which may result in a feedback loop by increasing IGF-I levels that act in an auto or paracrine manner to activate both, Ras and PI3K pathways. Together, these results support IGF-I signaling as an important growth and survival pathway in osteosarcoma cells.

Different miRNAs in osteosarcoma have been reported to target IGF-I signal transducers and transcription factors responsible for cellular invasion, epithelial to mesenchymal transition (EMT), stemness and chemoresistance. The IGF-IR itself is targeted by miR-133b, frequently downregulated in osteosarcomas (Zhao et al., 2013). PTEN is regulated by miR-93 and miR-23a, resulting in increased proliferation and migration (Kawano et al., 2015; Tian et al., 2015). PTEN is also the target of miR-144 in nasopharyngeal carcinomas (Zhang et al., 2013). However, this tumor suppressor-like behavior described in epithelial carcinomas has a reversed effect in osteosarcoma cells, where its downregulation is associated with cell proliferation and invasion by downregulating its target gene, TAGLN (Zhao et al., 2014). Besides, miR-144 may also regulate migration and invasion of osteosarcoma cells by targeting the cell adhesion protein Ezrin (Cui and Wang, 2015). FOXO1 is a transcriptional factor downstream of the PI3K pathway and a positive regulator of bone formation in osteoblasts (Rached et al., 2010). Interestingly, FOXO1 has recently been reported to be the target of miR-135b and miR-374, thus promoting proliferation and invasion in osteosarcomas (He et al., 2015; Pei et al., 2015). miR-101 and miR-155 have been shown to favor and to inhibit chemosensitivity by respectively blocking and inducing autophagy (Chang et al., 2014; Chen et al., 2014). These counteracting effects are somewhat controversial since both miRNAs target the same pathway, but at different levels: miR-101 downregulates mTOR expression levels (Merkel et al., 2010), a major regulator of autophagy together with AMPK, whereas miR-155 targets the alpha regulatory subunit of PI3K (p85), an upstream activator of mTOR (Huang et al., 2012). One plausible explanation could rely on the promiscuity of miRNAs so that the final outcome depends on other targets regulated by miR-101 and miR-155. In this regard, miR23a was also shown to target molecules other than PTEN that may enhance its oncogenic potential. Overexpression of miR-23a inhibited osteosarcoma HOS cells differentiation by downregulating connexin-43 (Cx43/GJA1), a mediator of intercellular signaling critical to osteoblast development (Gindin et al., 2015). In the same cluster as miR-23a, miR-27a promotes pulmonary osteosarcoma metastasis by downregulating the transcriptional regulator CBFA2T3 (Salah et al., 2015).

Here we demonstrate that miR-200c is upregulated in lung metastasis and ectopic expression of miR-200c in U2-OS cells increased cellular proliferation and migration but reduced basal phospho-AKT levels. In addition, multivariate analysis of immunohistochemical staining for signaling pathways showed a significant association of Akt activation with poorer prognosis. Based on the inhibitory role of miR200 family on Akt signaling, these results suggest that Akt and miR-200c contribute to osteosarcoma progression through converging mechanisms.

2. Materials and methods

2.1. Patients and samples

A retrospective study was performed in a primary cohort of 36 patients with primary osteosarcomas who underwent treatment between 1990 and 2011. These cases were collected from the archives of Hospital La Fe in Valencia. Clinical characteristics for each patient are indicated in Supplementary Table 1 and summarized in Table 1. For miRNA studies, nucleic acids were collected from 11 primary osteosarcomas plus 15 pulmonary metastases. A validation cohort of 16 patients was collected from the archives of the Pathology Department of the University of Valencia and University Clinic Hospital. The clinical characteristics of patients included in this second cohort are summarized in Supplementary Table 2. All samples from each cohort were reviewed independently by two expert pathologists to confirm histology and select tumor areas for tissue microarray construction and nucleic acid isolation. Written informed consent was signed by the patients; when not possible (dead or unreachable patients), the study material was used after decoding in accordance with Spanish law and with the approval of the Institutional Review Board. All procedures were done in accordance with the Helsinki declaration.

Table 1 – Summary of clinicopathologic features of 36 patients with primary osteosarcoma included in the immunohistochemical study.

Characteristics	n (%) *Median (range)
Age	14.47 (6.71–20.99)*
Gender	
Male	18 (50%)
Female	18 (50%)
Localized disease	33 (92%)
Site of primary disease	
Femur	20 (56%)
Tibia	8 (22%)
Humerus	3 (8%)
Fibula	1 (3%)
Pelvis	1 (3%)
Other	3 (8%)
Tumor sample analyzed	
Diagnostic biopsy (pre-chemotherapy)	16 (44%)
Surgery (post-chemotherapy)	20 (56%)
Treatment	
Metotrexate	34 (94%)
Ifosfamide	24 (67%)
Doxorrubicin	36 (100%)
Cisplatin	35 (97%)
VP-16	6 (17%)
Ciclophosfamide	16 (50%)
Type of surgery	
Conservative	25 (69%)
Radical	10 (28%)
Necrosis rate	70% (0–100%)*
Relapse/Progression	23 (64%)
Survivors	17 (47%)
Follow-up time (survivors)	11.11 years (2.1–26.4)*

2.2. Tissue microarray assembling and immunohistochemical analysis

Two 1 mm diameter cores were punched from each paraffin block using a manual tissue arrayer and mounted in an array paraffin block. Internal controls were also included in the tissue array. To correlate activated signaling pathways with clinical data, we selected phospho-AKT and phospho-ERK as two major druggable pathways activated by multiple signaling molecules with high relevance in osteosarcoma, including growth factors and tyrosine kinase receptors such as IGF-I and IGF-IR. We evaluated the activation of WNT/β-catenin (CTNNB1) pathway based on the fact that dysregulation of beta-catenin signaling is common in osteosarcoma and is implicated in the pathogenesis of osteosarcoma (Haydon et al., 2002). In addition, we included the evaluation of Sonic Hedgehog (SHH)/Gli1 activation in the immunohistochemical analysis because it is a druggable pathway and has been implicated in osteosarcoma (Lo et al., 2014).

3 μm Sections from the paraffin-embedded microarray were stained with the following antibodies: Akt-pS473, Phosphorylation Site Specific (Dako M3628); β-catenin (Dako M3539); phospho-Erk1,2 (Cell Signaling 4370); Gli1 (R&D Systems AF3324). For phospho-AKT, phospho-ERK and β-catenin both, cytosolic or nuclear stainings were considered as positive. For Gli1 only nuclear stainings were considered as positive. Immunohistochemistry was performed after confirming by hematoxylin/eosin staining that the samples in the tissue microarray were representative.

For immunoreactivity evaluation we used a previously reported semi-quantitative scoring system based on the overall stain intensity and percentage of neoplastic stained cells (Hatanaka et al., 2003). Briefly, the HSCORE was based on the percentage of neoplastic cells stained (Pi) and their staining intensity (i) and was calculated in each case according to the formula: HSCORE = $\sum$(i × Pi) where i = 0, 1, 2, 3 and Pi varies from 0 to 100%. Hence, the range for the HSCORE was 0–300. Each slide was evaluated separately by 2 independent pathologists (M.H. and M.L.) and mean value of both cores was calculated per tumor sample. In case of discrepancy between pathologists, slides were re-evaluated and consensus final score agreed. The Youden index was used as a *criterion* for selecting the optimum cut-off points. Cut-off values of 40 for either phosphor-AKT or β-catenin stainings were determined by ROC curve analysis. These data are provided in Supplementary Table 1.

2.3. miRNA isolation and array hybridization

Tumor areas were carefully selected avoiding adjacent normal or necrotic tissue. Five core punches were obtained from each FFPE block. AllPrep® FFPE kit (QIAGEN) was used to isolate RNA and DNA when possible. Samples were deparaffinized according to the manufacturer's instructions and proteinase K digested 24–48 h according to visual inspection tissue disaggregation. After RNA supernatant and DNA pellet separation, RNA was incubated at 80 °C to partially reverse formalin cross-linking, column purified and DNase digested. Total RNA was eluted in 14 μl H_2O. RNA purity and concentration were evaluated spectrophotometrically and fluorimetrically by NanoDrop ND-2000 (ThermoFisher) and Qubit (Invitrogen). Spectrophotometry measurements at three different wavelengths (230, 260 and 280 nm) and A260/230 and A260/280

ratios were used to assess the presence of contaminants: peptides, phenols, aromatic compounds, or carbohydrates and proteins. Qubit RNA HS kit was applied to highly degraded RNA samples to accurate quantitation of double stranded DNA and RNA respectively. Integrity and related size of RNA were assessed by microfluidics-based platform Agilent 2100 Bioanalyzer with RNA 6000 Nano Kit. Electropherograms were visualized at the Agilent 2100 Expert software including data collection, peak detection and interpretation of profiles.

RNA tumor samples were analyzed with miRNA Affymetrix platform miRNA 3.0 to obtain the miRNA and other non-coding RNA profile. 50 ng Of total RNA were added to the labeling mix following FlashTag miRNA (Genisphere) direct labeling manufacturer procedures. The GeneChip® Scanner 3000 7G System and reagents from Affymetrix were used to hybridize, wash, stain and scan the arrays. Expression console free software from Affymetrix was used for quality control and probe set normalized values assessment.

2.4. Statistical analysis

Data were summarized using mean, median, standard deviation and range in the case of continuous variables and with relative and absolute frequencies in the case of categorical variables. Differences between survival curves were assessed using the Log rank test. Signaling pathways found to be significant were evaluated with multivariable Cox regression analysis. Differentially expressed miRNAs between primary and metastatic tumors were assessed using an Elastic Net penalized logistic regression model. Differences between primary and metastatic tumor for each miRNAs selected for validation were assessed using the Wilcoxon–Mann Whitney test. p values <0.05 were considered statistically significant. All analyses were performed using R (version 3.2.2).

2.5. Tissue culture and transfection conditions

U2-OS cells were grown in DMEM media supplemented with 10% fetal bovine serum plus antibiotics. One day before transfection cells were trypsinized, counted and 10^5 cells per well were seeded in 6 well plates. On the day of the transfection media was replaced with 1 ml of media without serum and antibiotics. miR-200c mimic (Sigma) was diluted in 150 μl Opti-MEM media (Invitrogen) and mixed with 150 μl of Opti-MEM containing 9 μl of RNAiMAX (Invitrogen). 250 μl Of the mixture containing miR-200c mimic was added drop by drop to the cells to a final concentration of 15 nM. Eight hours post-transfection media was replaced with regular fresh growing media.

Twenty four hours post-transfection cells were trypsinized and counted before using them in migration and proliferation assays. For wound healing assays 2×10^5 cells were seeded in triplicates in Cytoselect™ 24-well plates. One day later the insert was carefully removed and healing of the wound was monitored. Pictures were taken every 2 h with an inverted microscope. For proliferation assays, 10^3 or 3×10^3 cells were seeded in triplicates in 96-well plates. Colorimetric assay with XTT (Roche) was determined at the indicated days following manufacturer's instructions for use.

In parallel, miRNA was isolated with miRNeasy Mini Kit (QIAGEN) two days after the transfection and miR-200c-3p (Applied biosystems No. 002300, Cat. 442797) and RNU6B (Applied biosystems No. 001093 Cat. 4427975) as endogenous control were quantified by real-time PCR using TaqMan microRNA assays (Applied biosystems).

3. Results

3.1. Identification of prognostic signaling pathways in primary osteosarcomas

To identify druggable pathways that correlate with poor prognosis in child and adolescent osteosarcomas, we conducted a multivariate immunohistochemical analysis in a cohort of 36 primary osteosarcoma cases (see Table 1 for clinical variables). Immunostaining analysis for the activation of the PI3K-AKT pathway was determined with pAKT (Ser473) antibodies and revealed a broad variability in both, the intensity and the distribution pattern (Figure 1A). Evaluation of the immunostainings by H-score revealed a significant correlation between activated AKT and poorer prognosis in primary osteosarcomas before chemotherapy (Figure 1B), supporting the role of this pathway in osteosarcoma progression. In contrast, no significant correlation was found when the analysis was done with 20 tumor samples obtained after chemotherapy (Figure 1C), suggesting that tumor response to chemotherapy may result in temporal or permanent loss of AKT activation and therefore, tissues samples obtained post-chemotherapy may not be appropriate for phospho-AKT analysis to predict outcome. Hence, these data support a role for AKT activation in osteosarcoma progression and suggest that immunohistochemical analysis of pre-chemotherapy biopsies is a powerful predictive tool to carefully-select pediatric patients for treatment with inhibitors of this pathway as a strategy to improve outcome.

We also evaluated the role of WNT signaling pathway in osteosarcoma prognosis by analyzing the cytosolic/nuclear expression of CTNNB1 (β-catenin). Cytosolic staining was scored as positive since increased cellular cytosolic β-catenin levels have been associated with increased β-catenin target protein expression (Sellin et al., 2001); most non-membrane expression of β-catenin is cytosolic in breast cancer and is associated with poor outcome (Lopez-Knowles et al., 2010). Only 28% of the osteosarcomas expressed β-catenin (Supplementary Figure 1A), somewhat lower than pAKT positive cases (44%). Interestingly, positive immunostaining for β-catenin only correlated with lower overall survival in osteosarcoma primary tumors after chemotherapy (Supplementary Figure 1B) but not in pre-chemotherapy tumors alone (Supplementary Figure 1C). However, β-catenin positivity was not strong enough to be considered an independent variable (Supplementary Table 3), perhaps reflecting the need for additional cases in the cohort.

3.2. Differential expression of miRNAs between primary osteosarcomas and lung metastases in the primary cohort

Increasing evidence supports the role of miRNAs in cancer etiology and progression. To identify targets that may regulate osteosarcoma progression, we conducted a high-throughput screening of miRNAs by comparing primary tumors vs. resected lung metastases. In this first cohort, 11 primary

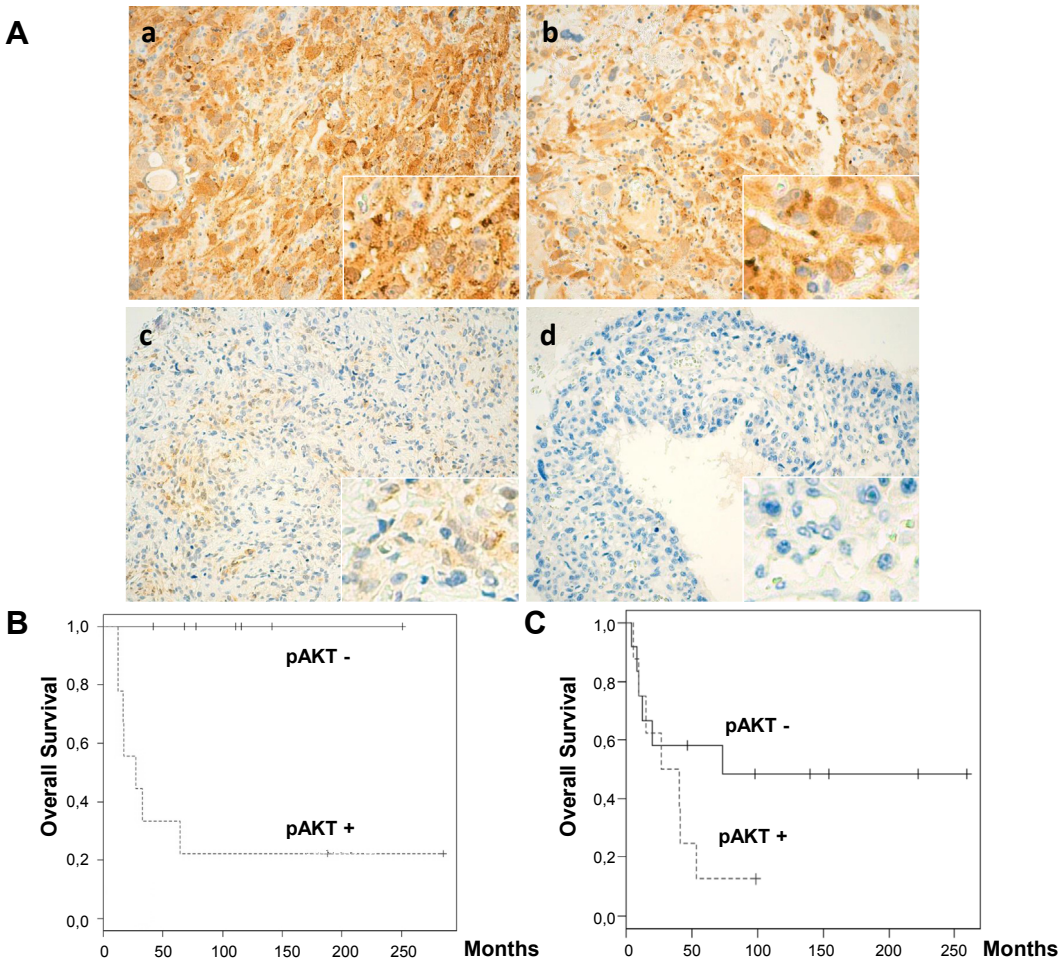

Figure 1 – Akt activation correlates with lower overall survival in primary osteosarcomas. (A) Representative immunostaining with phosphor-AKT (Ser473) antibodies showing variability from 95% of stained osteosarcoma cells with 3+ intensity (a) to 0% of stained cells (d). Inlets in the bottom right corner are magnifications to show the details of the stainings. (B) Kaplan–Meier survival curves in 16 primary osteosarcomas before chemotherapy; (Log Rank test, p = 0.003). (C) Kaplan–Meier survival curves in 20 primary osteosarcoma cases obtained after chemotherapy; (Log Rank test, p = 0.173).

tumors were compared to 15 lung metastases, all from La Fe hospital. Punches from paraffin-embedded tissues were selected carefully by pathologists to avoid contamination by non-tumorigenic tissue. Using Elastic Net to perform a penalized logistic regression analysis of the microarray data, we generated a heatmap model of 26 miRNAs that were differentially expressed between primary and metastatic tumors (Figure 2A). Of these, only means for mirR200c, miR4786-3p and ENSG00000212490_x displayed at least a two-fold difference and were thus selected for further validation.

3.3. *Differential expression of miRNA between primary osteosarcomas and lung metastases in the validation cohort*

A second cohort of 10 primary tumors and 6 metastases from a different hospital (University Hospital, Valencia) were used to validate the results of the miRNAs in osteosarcoma metastases. In this second analysis, only expression of miR-200c was significantly changed between primary and metastatic osteosarcomas (Figure 2B). Collectively, these results strongly support a role for miR200c in the molecular processes of lung metastasis. Interestingly, expression analysis revealed a strong correlation between miR200c and either miR-375 or miR141 (Figure 3); these particular miRNAs are involved in EMT as well as in a negative regulation of PI3K/AKT pathway. Expression of miR-30a also displayed a correlation with miR200c, but to a lower extent than miR-375 and miR-141 ($R = 0.73$, $p = 0.008$, data not shown), suggesting a role in immunosuppressive functions as well as in mesenchymal to epithelial transition (MET) and in inhibition of osteogenic differentiation. We did not detect an inverse correlation between the expression of any miRNAs and pAKT, β-catenin, or other clinical parameters except lung metastases. This lack of correlation between miRNAs and activated AKT may reflect the need for increasing the n numbers of osteosarcoma cases or alternatively, these may simply be unrelated variables. Thus, although we cannot yet conclude whether miR-200c and pAKT are regulated by independent pathways, our results clearly demonstrate that both molecules have significant value in predicting osteosarcoma progression.

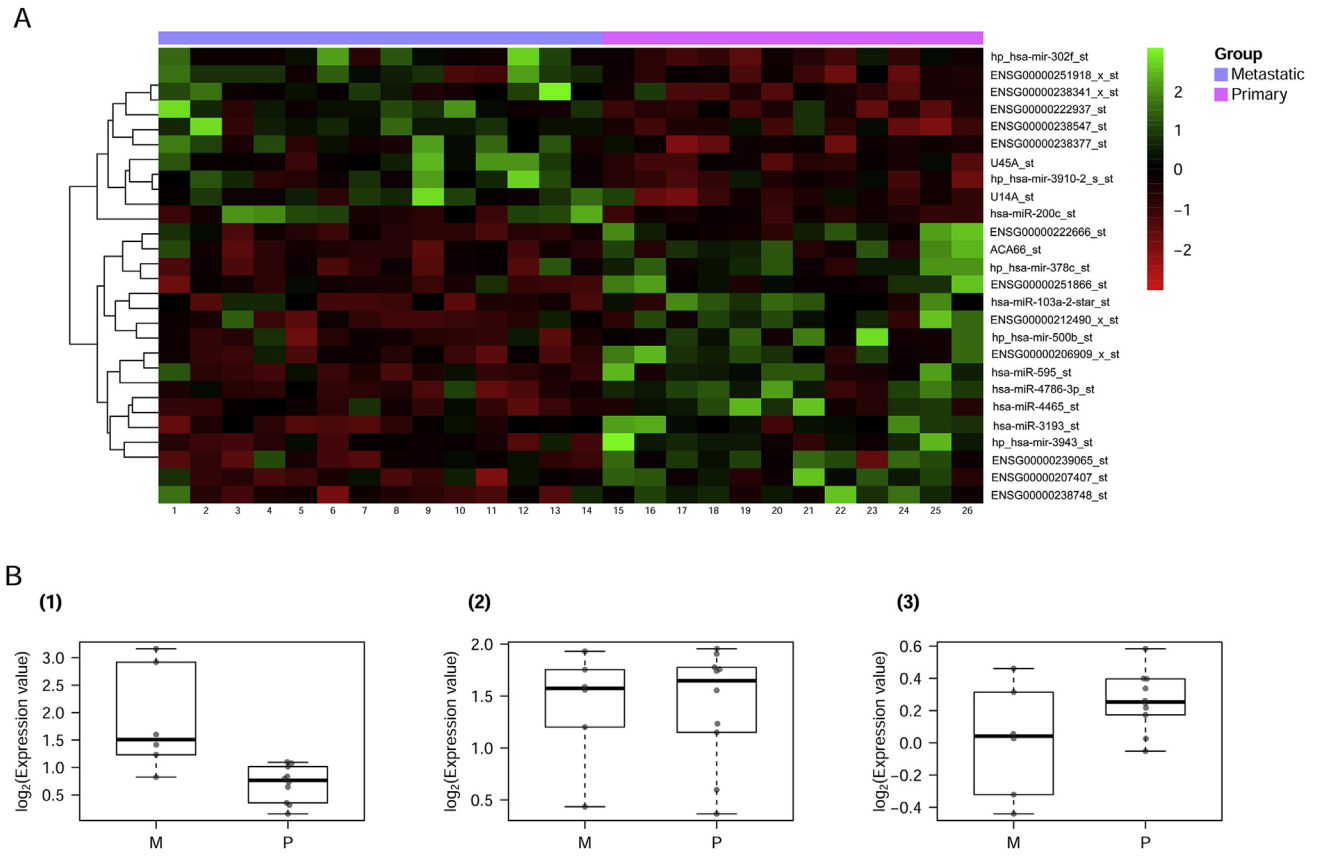

Figure 2 – miR-200c expression associates with osteosarcoma pulmonary metastasis. (A) Heatmap of selected miRNAs by the Elastic Net regression model in a cohort of 26 osteosarcomas. Each row shows data for a specific miRNA in every osteosarcoma case and each column represents the miRNA expression profile for every osteosarcoma. Rows have been ordered according to the results of the Ward hierarchical clustering algorithm. Specific values in each row have been standardized to z-scores (scale on the top right) based on the variable means for each miRNA. Primary or metastatic condition for the osteosarcoma cases are indicated above the heatmap. (B) miRNA validation in a new cohort of 16 osteosarcomas. Boxplots of differential expression between 6 primary (P) and 10 metastatic (M) osteosarcomas in (1) mirR200c, $p = 0.003$; (2) miR4786-3p, $p = 0.96$; and (3) ENSG00000212490_x, $p = 0.23$.

3.4. *Exogenous expression of miR-200c in osteosarcoma cells enhances cellular migration and proliferation*

In contrast to epithelial cancer cells where miR-200c expression blocks EMT, proliferation, and metastasis (Gregory et al., 2008; Hur et al., 2013; Ibrahim et al., 2015; Liu et al., 2014; Song et al., 2015; Tang et al., 2013; Yu et al., 2010), our analysis of osteosarcoma tumors indicate a robust correlation between miR-200c expression and metastasis. To further test this novel paradigm, we performed a series of *in vitro* experiments with U2-OS cells, a human cell line originally derived from a primary osteosarcoma. Transfection of miR-200c mimic into U2-OS cells increased significantly cellular migration (Figure 4). We next evaluated the role of miR-200c in cellular proliferation by analyzing the growth of U2-OS cells. Cultures transfected with miR-200c mimic displayed a higher proliferation ratio than controls (Figure 5A). Moreover, the enhanced proliferation correlated with higher expression levels of cyclin A expression (Figure 5B), a hallmark of proliferative cells. Interestingly, miR-200c mimic transfection resulted in lower basal pAKT levels in U2-OS cells, a result that also supports its long-term role in MET. Based on this result, it is possible that pAKT and miR-200c are inversely correlated *in vivo* in osteosarcoma tumors. However, using our cohorts of patients, we could not establish a significant correlation between pAKT and miR-200c ($R = -0.207$, $p = 0.34$), perhaps owing to the reduced number of cases where data for both biomarkers was available ($n = 23$). Alternatively, pAKT and miR-200c may reflect two distinct mechanisms which culminate in metastasis: cells that use pAKT pathway for metastasis do not necessarily require miR-200c expression, whereas cells that use miR-200c overexpression will display reduced pAKT.

3.5. *Pulmonary osteosarcoma metastases express epithelial marker E-cadherin*

Based on these results, our working hypothesis is that overexpression of miR-200c is one of the mechanisms underlying lung metastasis in osteosarcoma. To test this hypothesis, we analyzed E-cadherin expression in metastatic osteosarcoma tumors. As expected, some cells within each tumor stained positive for E-cadherin (Figure 5C). Immunohistochemistry may not be sensitive enough to detect E-cadherin in cells

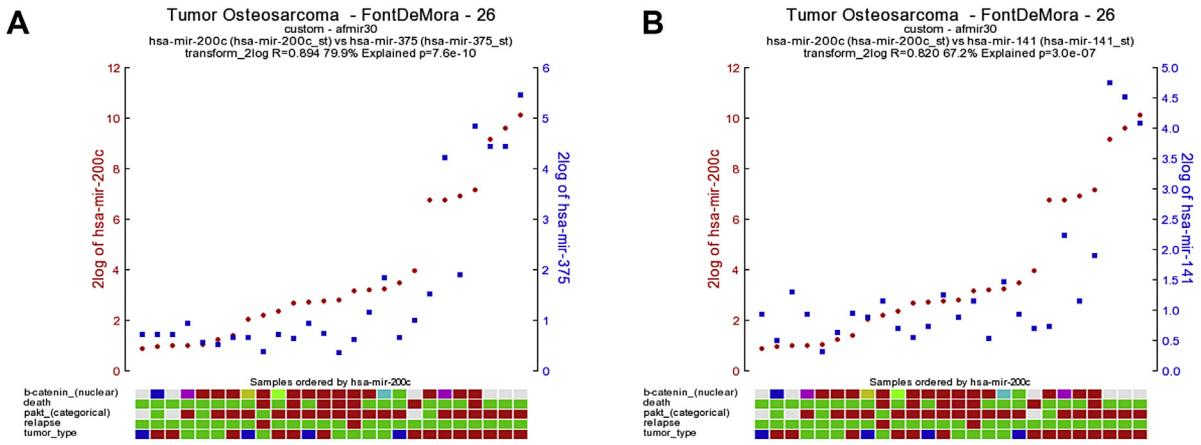

Figure 3 – Correlation between the expression levels of miR-200c and miR-375 or miR-141 in osteosarcomas. Red dots represent the expression levels of miR-200c and blue dots represent the expression levels of (A) miR-375 ($R = 0.894$, $p < 0.001$), or (B) miR-141($R = 0.82$, $p < 0.001$). Each pair of red-blue dots represents a patient for which clinical and histological data are indicated at the bottom. Data was uploaded and analyzed in R2: microarray analysis and visualization platform (http://r2.amc.nl) revealed a very strong correlation between the expression of miR-200c with (A) miR-375 and (B) miR-141.

with low levels of expression which require other techniques such as RT-PCR or microarray analysis. Indeed, *in silico* analysis using Guenther expression data available at R2 (http://r2.amc.nl) revealed a strong increase of E-cadherin expression in osteosarcoma lung metastases vs. primary tumors (Figure 5D), further supporting our hypothesis that osteosarcomas frequently undergo MET to metastasize to the lung.

4. Discussion

In the present study, we compared expression patterns in primary osteosarcomas with lung metastases in an effort to identify molecular pathways that potentially mediate metastasis of this cancer. Our findings reveal that phosphorylated AKT is enhanced in primary tumors whereas expression of miR-200c is upregulated in lung metastases. Relapsed osteosarcoma is characterized by complex genes and signaling pathways driving its development and metastasis (Moriarity et al., 2015). Among the different pathways, activation of AKT has been implicated in pulmonary metastasis using cell line models of osteosarcoma (Fukaya et al., 2005). However, to our knowledge, this is the first report that activation of AKT correlates with outcome in primary human osteosarcoma tumors. PI3K-AKT signaling pathway regulates pleiotropic effects in the cell and is a major target to prevent progression. Upstream targets such as FGFR1 (Weekes et al., 2015) or IGF-IR (Wang et al., 2012) have also suggested to be efficient therapies in osteosarcoma. Nevertheless, targeted therapy combining PI3K-AKT inhibitors plus other drugs to match additional alterations present in the patient was not sufficient to prevent progression (Subbiah et al., 2015). Therefore, other intracellular signaling pathways not explored before are critical for the pulmonary metastasis of osteosarcoma.

The findings of the present study demonstrate a new role for miR-200c in mediating the lung metastasis associated with osteosarcoma. Our findings contrast the published role of miR-200c in tumors of epithelial origin where it functions as a tumor suppressor by inhibiting epithelial to mesenchymal transition (EMT) through the downregulation of ZEB1 (Burk et al., 2008; Gregory et al., 2008; Park et al., 2008). In two independent cohorts of osteosarcoma patients, we observed that miR-200c is overexpressed in lung metastasis. In addition to ZEB1, miR-200c also targets the transcription factors BMI1, E2F3 (Liu et al., 2014) and SIP1 (Gregory et al., 2008), all of which are involved in regulation of EMT. Given the apparent contrast of its expression pattern between epithelial tumors and lung metastasis, overexpressed miR-200c may facilitate osteosarcoma progression by promoting MET rather than EMT in tumor cells. Interestingly, expression of miR-141, another member of the miR-200 family that clusters with miR-200c in chromosome 12p13.31, also correlated with miR-200c expression in our studies of pulmonary metastasis. This result suggests a common regulatory element shared by both miRNAs in the regulation of cellular differentiation. Based on the emerging evidence that supports a role for miR-200c in promoting EMT, our results suggest the novel concept that miR-200c facilitates osteosarcoma metastasis by facilitating the reverse process of MET. These results may also explain why osteosarcomas metastasize mainly in the epithelial niche of lungs (where nearly any cancer can spread) and not in bone marrow or other sites.

The WNT/β-catenin pathway has been associated with bone cancers (reviewed in Tian et al., 2014) and its upregulation promotes EMT in osteosarcoma cells (Lv et al., 2016). In the present study, we demonstrate that activation of β-catenin in primary osteosarcomas after chemotherapy associates with poorer prognosis. Both AKT and β-catenin activation promote EMT and are prognostic biomarkers in primary tumors. However, many lung metastases show increased expression of the epithelial markers CDH1 and miR-200c. These results suggest that during osteosarcoma progression a shift to MET occurs, probably due to the gain of additional genetic or epigenetic alterations.

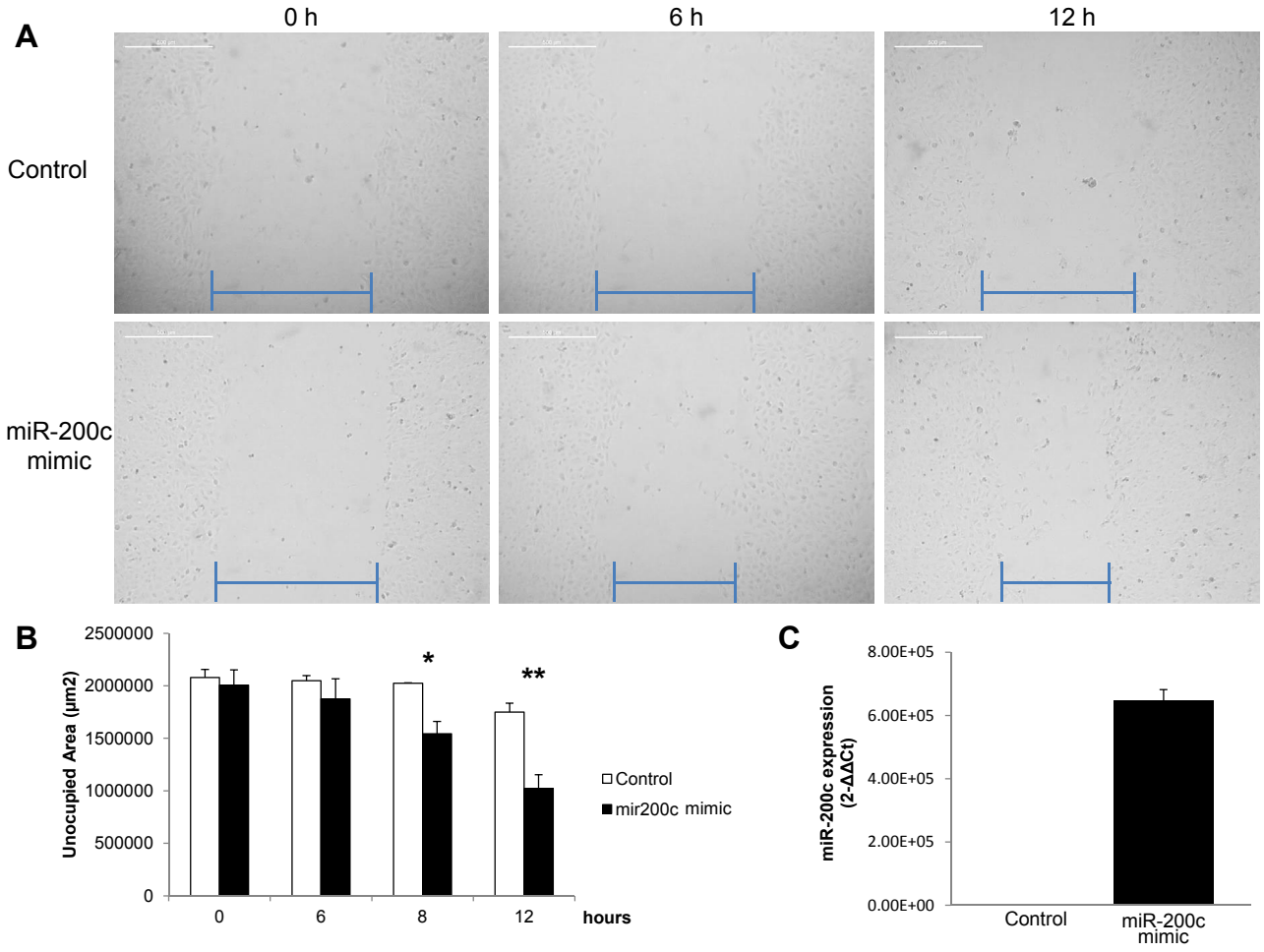

Figure 4 – Expression of miR-200c mimic in U2-OS cells enhances cellular migration. (A) Representative images of U2-OS cells transfected with miR-200c mimic or control. Images were taken at the indicated times after removing the wound field inserts. Migration was measured by wound healing assay. White bars correspond to 500 µm. Bottom bars indicate the average unoccupied distance for each image. (B) Unoccupied areas corresponding to wound fields were measured on triplicate images using *ImageTool* v3.0 software. Bars correspond to means of three independent experiments ± standard error. *p = 0.03; **p = 0.04. (C) Quantitative PCR analysis of U2-OS cells to show the efficiency of the transfection with mir200c two days later.

One potential explanation for the increased expression of miR-200c in the lung metastasis as compared to primary tumors is the possibility that miR200c appears late in osteosarcoma progression. Therefore, circulating cells derived from the primary tumor would contain high levels of miR-200c and this enhanced expression would favor their retention and growth in the lung. A recent analysis of pre-chemotherapy biopsy samples reported expression of miR-27a and miR-181c* in patients who later developed clinical metastatic disease (Jones et al., 2012). However, miR-200c expression was not detected in these primary tumors, supporting our hypothesis that miR200c expression occurs during later stages of osteosarcoma progression. In our study, miR-200c was overexpressed in 50% of all metastases, perhaps indicating that molecules other than miR-200c also regulate osteosarcoma progression. Further studies will be needed to elucidate additional mechanisms that regulate lung metastases. We cannot exclude the remote possibility that detection of miR200c expression in lung metastases is the result of epithelial contamination by pulmonary cells. However, the areas of the tumor used for miRNAs analysis were carefully selected by skilled pathologists in each cohort. Additionally, 6 out of 12 primary osteosarcomas also displayed miR200c expression (see middle samples with green and blue dots under "tumor_type" in Figure 5), suggesting that its detection is not exclusive to lung metastases. Moreover, lung metastases express higher E-cadherin levels than primary osteosarcomas (Figure 5D), further supporting the notion that osteosarcoma metastases undergo MET for which miR200c expression is required.

We also found a strong correlation between miR-200c expression and miR-375 in osteosarcomas. miR-375 negatively regulates PDK-1 expression and PI3K-AKT signaling (Garikipati et al., 2015). Interestingly, miR-375 was able to inhibit osteogenic differentiation via the regulation of RUNX2 expression (Du et al., 2015). Several experimental approaches have led to the proposal that RUNX2 expression in breast cancers may explain their metastatic preference for bone (Taipaleenmaki et al., 2015; Zhang et al., 2015). Following

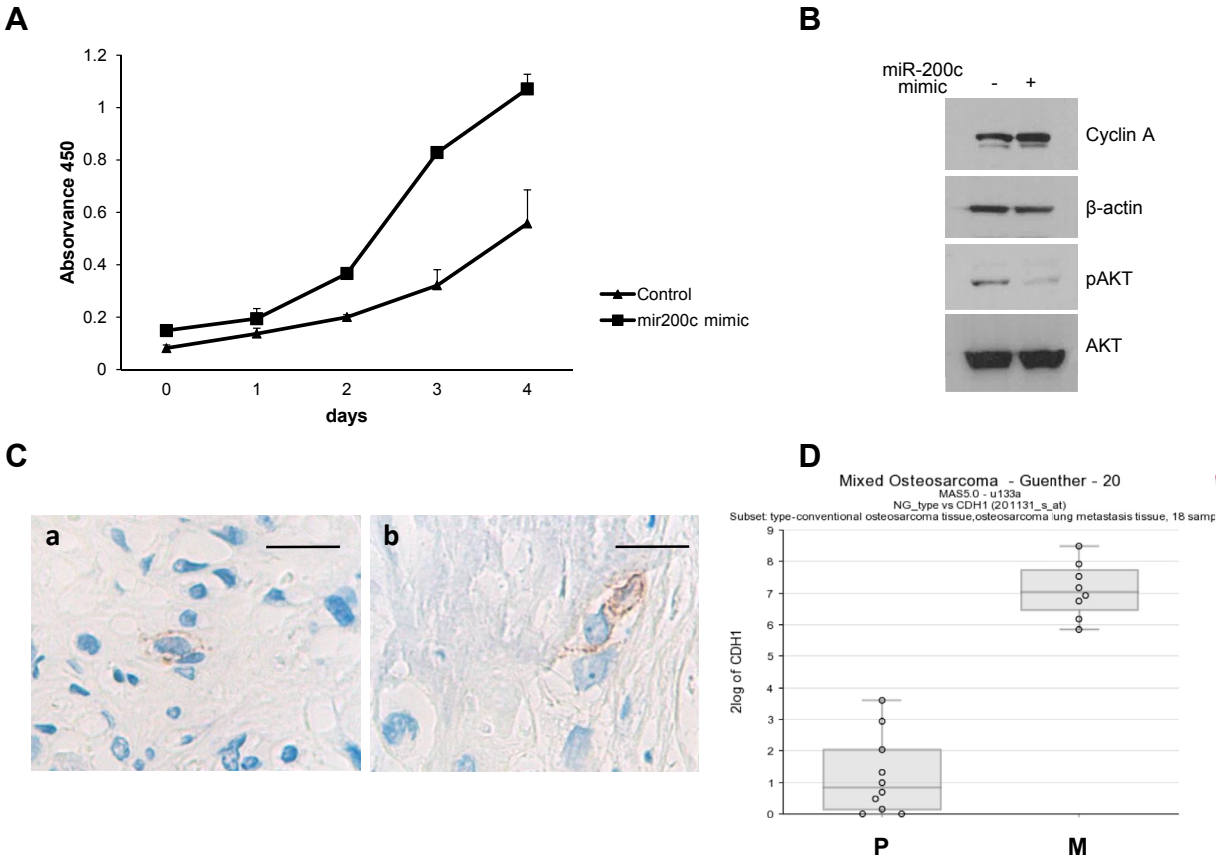

Figure 5 – miR-200c mimic increases proliferation ratio in U2-OS cells. (A) U2-OS cells were transfected with miR-200c mimic or control and next day cells were collected and seeded at 10^3 cells per well in 96 well plates. Proliferation was determined at the indicated times by XTT colorimetric assay. Bars correspond to means of three independent experiments ± standard error. (B) Western blot analysis with the indicated antibodies of U2-OS cells transfected two days before with miR-200c mimic or control. (C) Immunohistochemical staining for E-cadherin reveals a weak and heterogeneous expression in lung metastatic cells. Centered cells in (a) and (b) show membrane expression of E-cadherin in two different tumors with highest miR-200c expression (see Figure 3). Bars correspond to 20 μm. (D) Boxplot representation of E-cadherin (CDH1) differential expression between primary osteosarcomas (P) and lung metastases (M). One way analysis of variance (ANOVA, $p < 0.001$). Total RNA was isolated from osteosarcoma tumors and used to hybridize Affymetrix arrays (GeneChip Human Genome u133a array). Data obtained from Guenther study at R2: microarray analysis and visualization platform (http://r2.amc.nl).

this line of reasoning, it is also possible that miR-375 expression in osteosarcoma may reduce preferences for bone metastases by targeting RUNX2. Therefore, miR-200c, miR-141 and miR-375 may promote lung metastasis by regulating different pathways in osteosarcoma cells. Collectively, our results support the idea that miR-200c signaling is a potential therapeutic target for preventing osteosarcoma metastasis and therefore, combination therapy directed against the PI3K-AKT-mTOR pathway and the miR-200c pathway may constitute a more efficient strategy for blocking osteosarcoma progression.

Acknowledgments

We are very grateful to Empar Mayordomo for helpful discussion with immunostainings and Almudena Vidal for technical assistance. We are also grateful to María Pilar Marín at Microscopy Unit IIS La Fe for acquisition of images. We are thankful for José Luis Zugaza for reviewing the manuscript. Pablo Berlanga was supported by a post-resident grant by IIS La Fe. This study was funded by grant SAF2012-37330 from Spanish Ministry of Economy and Competitivity.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2016.04.004.

REFERENCES

Arndt, C.A., Rose, P.S., Folpe, A.L., Laack, N.N., 2012. Common musculoskeletal tumors of childhood and adolescence. Mayo Clin. Proc. 87, 475–487.

Burk, U., Schubert, J., Wellner, U., Schmalhofer, O., Vincan, E., Spaderna, S., Brabletz, T., 2008. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. 9, 582–589.

Cui, S.Q., Wang, H., 2015. MicroRNA-144 inhibits the proliferation, apoptosis, invasion, and migration of osteosarcoma cell line F5M2. Tumour Biol. 36, 6949–6958.
Chang, L., Shrestha, S., LaChaud, G., Scott, M.A., James, A.W., 2015. Review of microRNA in osteosarcoma and chondrosarcoma. Med. Oncol. 32, 613.
Chang, Z., Huo, L., Li, K., Wu, Y., Hu, Z., 2014. Blocked autophagy by miR-101 enhances osteosarcoma cell chemosensitivity in vitro. Sci. World J. 2014, 794756.
Chen, L., Jiang, K., Jiang, H., Wei, P., 2014. miR-155 mediates drug resistance in osteosarcoma cells via inducing autophagy. Exp. Ther. Med. 8, 527–532.
Choy, E., Hornicek, F., MacConaill, L., Harmon, D., Tariq, Z., Garraway, L., Duan, Z., 2012. High-throughput genotyping in osteosarcoma identifies multiple mutations in phosphoinositide-3-kinase and other oncogenes. Cancer 118, 2905–2914.
Du, F., Wu, H., Zhou, Z., Liu, Y.U., 2015. microRNA-375 inhibits osteogenic differentiation by targeting runt-related transcription factor 2. Exp. Ther. Med. 10, 207–212.
Ferrero, M., Avivar, A., Garcia-Macias, M.C., Font de Mora, J., 2008. Phosphoinositide 3-kinase/AKT signaling can promote AIB1 stability independently of GSK3 phosphorylation. Cancer Res. 68, 5450–5459.
Fukaya, Y., Ishiguro, N., Senga, T., Ichigotani, Y., Sohara, Y., Tsutsui, M., Shioura, T., Iwamoto, T., Hamaguchi, M., 2005. A role for PI3K-Akt signaling in pulmonary metastatic nodule formation of the osteosarcoma cell line, LM8. Oncol. Rep. 14, 847–852.
Garikipati, V.N., Krishnamurthy, P., Verma, S.K., et al., 2015. Negative regulation of miR-375 by Interleukin-10 enhances bone marrow-derived progenitor cell-mediated myocardial repair and function after myocardial infarction. Stem Cells 33, 3519–3529.
Geng, S., Wang, X., Xu, X., Zhang, H., Ma, Y., Zhang, Y., Li, B., Bi, Z., Yang, C., 2014. Steroid receptor co-activator-3 promotes osteosarcoma progression through up-regulation of FoxM1. Tumour Biol. 35, 3087–3094.
Gindin, Y., Jiang, Y., Francis, P., Walker, R.L., Abaan, O.D., Zhu, Y.J., Meltzer, P.S., 2015. miR-23a impairs bone differentiation in osteosarcoma via down-regulation of GJA1. Front. Genet. 6, 233.
Gregory, P.A., Bert, A.G., Paterson, E.L., Barry, S.C., Tsykin, A., Farshid, G., Vadas, M.A., Khew-Goodall, Y., Goodall, G.J., 2008. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat. Cell Biol. 10, 593–601.
Han, G., Wang, Y., Bi, W., 2012. C-Myc overexpression promotes osteosarcoma cell invasion via activation of MEK-ERK pathway. Oncol. Res. 20, 149–156.
Hatanaka, Y., Hashizume, K., Nitta, K., Kato, T., Itoh, I., Tani, Y., 2003. Cytometrical image analysis for immunohistochemical hormone receptor status in breast carcinomas. Pathol. Int. 53, 693–699.
Haydon, R.C., Deyrup, A., Ishikawa, A., et al., 2002. Cytoplasmic and/or nuclear accumulation of the beta-catenin protein is a frequent event in human osteosarcoma. Int. J. Cancer 102, 338–342.
He, W., Feng, L., Xia, D., Han, N., 2015. MiR-374a promotes the proliferation of human osteosarcoma by downregulating FOXO1 expression. Int. J. Clin. Exp. Med. 8, 3482–3489.
Huang, X., Shen, Y., Liu, M., Bi, C., Jiang, C., Iqbal, J., McKeithan, T.W., Chan, W.C., Ding, S.J., Fu, K., 2012. Quantitative proteomics reveals that miR-155 regulates the PI3K-AKT pathway in diffuse large B-cell lymphoma. Am. J. Pathol. 181, 26–33.
Hur, K., Toiyama, Y., Takahashi, M., Balaguer, F., Nagasaka, T., Koike, J., Hemmi, H., Koi, M., Boland, C.R., Goel, A., 2013. MicroRNA-200c modulates epithelial-to-mesenchymal transition (EMT) in human colorectal cancer metastasis. Gut 62, 1315–1326.
Ibrahim, F.F., Jamal, R., Syafruddin, S.E., Ab Mutalib, N.S., Saidin, S., MdZin, R.R., Hossain Mollah, M.M., Mokhtar, N.M., 2015. MicroRNA-200c and microRNA-31 regulate proliferation, colony formation, migration and invasion in serous ovarian cancer. J. Ovarian Res. 8, 56.
Jones, K.B., Salah, Z., Del Mare, S., et al., 2012. miRNA signatures associate with pathogenesis and progression of osteosarcoma. Cancer Res. 72, 1865–1877.
Kager, L., Zoubek, A., Potschger, U., et al., 2003. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. J. Clin. Oncol. 21, 2011–2018.
Kawano, M., Tanaka, K., Itonaga, I., Ikeda, S., Iwasaki, T., Tsumura, H., 2015. microRNA-93 promotes cell proliferation via targeting of PTEN in Osteosarcoma cells. J. Exp. Clin. Cancer Res. 34, 76.
Kolb, E.A., Kamara, D., Zhang, W., Lin, J., Hingorani, P., Baker, L., Houghton, P., Gorlick, R., 2010. R1507, a fully human monoclonal antibody targeting IGF-1R, is effective alone and in combination with rapamycin in inhibiting growth of osteosarcoma xenografts. Pediatr. Blood Cancer 55, 67–75.
Liu, L., Qiu, M., Tan, G., Liang, Z., Qin, Y., Chen, L., Chen, H., Liu, J., 2014. miR-200c inhibits invasion, migration and proliferation of bladder cancer cells through down-regulation of BMI-1 and E2F3. J. Transl. Med. 12, 305.
Lo, W.W., Wunder, J.S., Dickson, B.C., Campbell, V., McGovern, K., Alman, B.A., Andrulis, I.L., 2014. Involvement and targeted intervention of dysregulated Hedgehog signaling in osteosarcoma. Cancer 120, 537–547.
Lopez-Knowles, E., Zardawi, S.J., McNeil, C.M., Millar, E.K., Crea, P., Musgrove, E.A., Sutherland, R.L., O'Toole, S.A., 2010. Cytoplasmic localization of beta-catenin is a marker of poor outcome in breast cancer patients. Cancer Epidemiol. Biomarkers Prev. 19, 301–309.
Lv, Y.F., Dai, H., Yan, G.N., Meng, G., Zhang, X., Guo, Q.N., 2016. Downregulation of tumor suppressing STF cDNA 3 promotes epithelial-mesenchymal transition and tumor metastasis of osteosarcoma by the Wnt/GSK-3beta/beta-catenin/Snail signaling pathway. Cancer Lett. 373, 164–173.
Merkel, O., Hamacher, F., Laimer, D., et al., 2010. Identification of differential and functionally active miRNAs in both anaplastic lymphoma kinase (ALK)+ and ALK− anaplastic large-cell lymphoma. Proc. Natl. Acad. Sci. U.S.A 107, 16228–16233.
Moriarity, B.S., Otto, G.M., Rahrmann, E.P., et al., 2015. A Sleeping Beauty forward genetic screen identifies new genes and pathways driving osteosarcoma development and metastasis. Nat. Genet. 47, 615–624.
Park, S.M., Gaur, A.B., Lengyel, E., Peter, M.E., 2008. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev. 22, 894–907.
Pei, H., Jin, Z., Chen, S., Sun, X., Yu, J., Guo, W., 2015. MiR-135b promotes proliferation and invasion of osteosarcoma cells via targeting FOXO1. Mol. Cell Biochem 400, 245–252.
Rached, M.T., Kode, A., Xu, L., Yoshikawa, Y., Paik, J.H., Depinho, R.A., Kousteni, S., 2010. FoxO1 is a positive regulator of bone formation by favoring protein synthesis and resistance to oxidative stress in osteoblasts. Cell Metab. 11, 147–160.
Salah, Z., Arafeh, R., Maximov, V., Galasso, M., Khawaled, S., Abou-Sharieha, S., Volinia, S., Jones, K.B., Croce, C.M., Aqeilan, R.I., 2015. miR-27a and miR-27a* contribute to metastatic properties of osteosarcoma cells. Oncotarget 6, 4920–4935.
Savage, S.A., Mirabello, L., 2011. Using epidemiology and genomics to understand osteosarcoma etiology. Sarcoma 2011, 548151.

Sellin, J.H., Umar, S., Xiao, J., Morris, A.P., 2001. Increased beta-catenin expression and nuclear translocation accompany cellular hyperproliferation *in vivo*. Cancer Res. 61, 2899–2906.

Song, C., Liu, L.Z., Pei, X.Q., Liu, X., Yang, L., Ye, F., Xie, X., Chen, J., Tang, H., Xie, X., 2015. miR-200c inhibits breast cancer proliferation by targeting KRAS. Oncotarget 6, 34968–34978.

Su, Y., Wagner, E.R., Luo, Q., et al., 2011. Insulin-like growth factor binding protein 5 suppresses tumor growth and metastasis of human osteosarcoma. Oncogene 30, 3907–3917.

Subbiah, V., Wagner, M.J., McGuire, M.F., Sarwari, N.M., Devarajan, E., Lewis, V.O., Westin, S., Kato, S., Brown, R.E., Anderson, P., 2015. Personalized comprehensive molecular profiling of high risk osteosarcoma: implications and limitations for precision medicine. Oncotarget 6, 40642–40654.

Taipaleenmaki, H., Browne, G., Akech, J., Zustin, J., van Wijnen, A.J., Stein, J.L., Hesse, E., Stein, G.S., Lian, J.B., 2015. Targeting of Runx2 by miR-135 and miR-203 impairs progression of breast cancer and metastatic bone disease. Cancer Res. 75, 1433–1444.

Tang, H., Deng, M., Tang, Y., Xie, X., Guo, J., Kong, Y., Ye, F., Su, Q., Xie, X., 2013. miR-200b and miR-200c as prognostic factors and mediators of gastric cancer cell progression. Clin. Cancer Res. 19, 5602–5612.

Tian, J., He, H., Lei, G., 2014. Wnt/beta-catenin pathway in bone cancers. Tumour Biol. 35, 9439–9445.

Tian, K., Di, R., Wang, L., 2015. MicroRNA-23a enhances migration and invasion through PTEN in osteosarcoma. Cancer Gene Ther. 22, 351–359.

Wang, Y.H., Han, X.D., Qiu, Y., et al., 2012. Increased expression of insulin-like growth factor-1 receptor is correlated with tumor metastasis and prognosis in patients with osteosarcoma. J. Surg. Oncol. 105, 235–243.

Weekes, D., Kashima, T.G., Zandueta, C., et al., 2015 Sep 21. Regulation of osteosarcoma cell lung metastasis by the c-Fos/AP-1 target FGFR1. Oncogene. http://dx.doi.org/10.1038/onc.2015.344 [Epub ahead of print].

Yu, J., Ohuchida, K., Mizumoto, K., Sato, N., Kayashima, T., Fujita, H., Nakata, K., Tanaka, M., 2010. MicroRNA, hsa-miR-200c, is an independent prognostic factor in pancreatic cancer and its upregulation inhibits pancreatic cancer invasion but increases cell proliferation. Mol. Cancer 9, 169.

Yu, Y., Luk, F., Yang, J.L., Walsh, W.R., 2011. Ras/Raf/MEK/ERK pathway is associated with lung metastasis of osteosarcoma in an orthotopic mouse model. Anticancer Res. 31, 1147–1152.

Zhang, L.Y., Ho-Fun Lee, V., Wong, A.M., et al., 2013. MicroRNA-144 promotes cell proliferation, migration and invasion in nasopharyngeal carcinoma through repression of PTEN. Carcinogenesis 34, 454–463.

Zhang, X., Akech, J., Browne, G., Russell, S., Wixted, J.J., Stein, J.L., Stein, G.S., Lian, J.B., 2015. Runx2-Smad signaling impacts the progression of tumor-induced bone disease. Int. J. Cancer 136, 1321–1332.

Zhao, H., Li, M., Li, L., Yang, X., Lan, G., Zhang, Y., 2013. MiR-133b is down-regulated in human osteosarcoma and inhibits osteosarcoma cells proliferation, migration and invasion, and promotes apoptosis. PLoS One 8, e83571.

Zhao, M., Huang, J., Gui, K., Xiong, M., Cai, G., Xu, J., Wang, K., Liu, D., Zhang, X., Yin, W., 2014. The downregulation of miR-144 is associated with the growth and invasion of osteosarcoma cells through the regulation of TAGLN expression. Int. J. Mol. Med. 34, 1565–1572.

1. INTRODUCCIÓN

1.1. El cáncer en la infancia y adolescencia

El cáncer en niños y adolescentes supone únicamente el 2% de todos los casos de cáncer que se diagnostican anualmente. Los avances en los tratamientos antineoplásicos y de soporte han supuesto un importante aumento de la supervivencia en las últimas décadas, alcanzando tasas de curación del cáncer infantil del 70-80% a los 5 años del diagnóstico. Sin embargo, a pesar de este aumento de supervivencia, el cáncer en la edad pediátrica continúa siendo la principal causa de mortalidad por enfermedad en los niños mayores de un año y el pronóstico continúa siendo sombrío en algunos tipos de cáncer (Figura 1) [1].

Desde el punto de vista epidemiológico, los distintos tipos de cáncer en la infancia se agrupan en 12 grupos diagnósticos principales siguiendo la International Classification of Childhood Cancer (ICCC-3) [2]: (I) leucemias, (II) linfomas, (III) tumores sistema nervioso central, (IV) neuroblastomas, (V) retinoblastomas, (VI) tumores renales, (VII) tumores hepáticos, (VIII) tumores óseos, (IX) sarcomas de partes blandas, (X) tumores de células germinales, (XI) melanomas/otras neoplasias epiteliales y (XII) otras neoplasias.

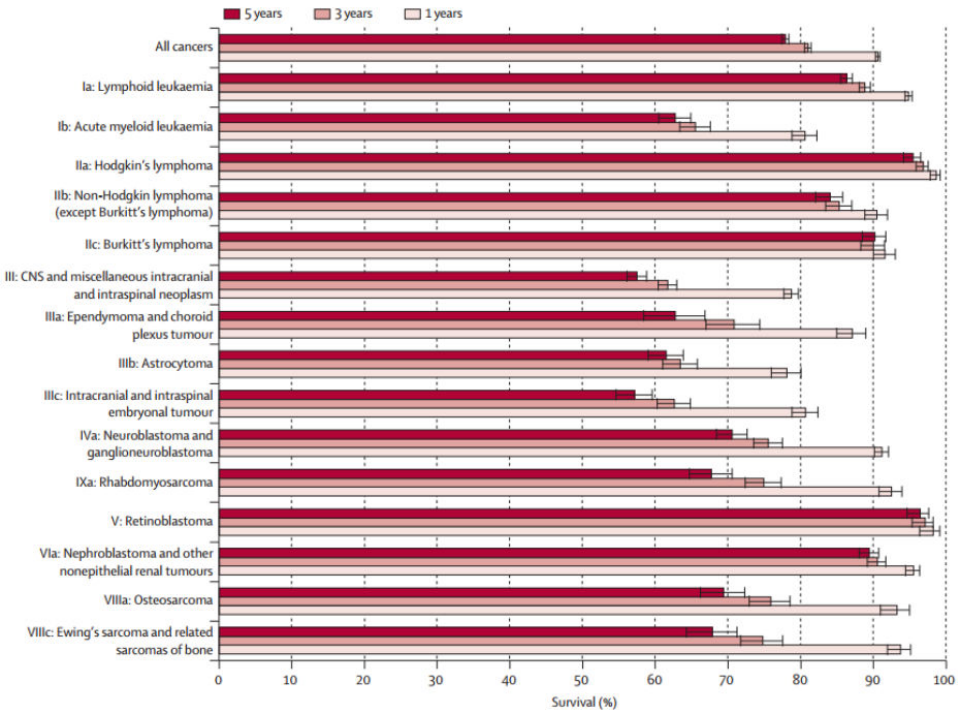

Figura 1: Supervivencia por grupo diagnóstico ICCC-3 de todos los tumores malignos (Pacientes 0-14 años población europea, Período: 2000-2007, n = 57.956 casos) [1]

1.2. Osteosarcoma

1.2.1. Introducción

El osteosarcoma es el tumor óseo maligno primario más frecuente en niños y adolescentes, así como uno de los tumores pediátricos con peor pronóstico [1]. La incidencia de osteosarcoma en la población general es de 2-3 casos/1.000.000/año llegando a los 8-11casos/1.000.000/año en la adolescencia [3]. Presenta una incidencia bimodal, con un pico de mayor incidencia en la adolescencia y otro pico después de los 65 años, principalmente secundario a radioterapia o enfermedad de Paget.

1.2.2. Histopatología

El osteosarcoma se caracteriza desde el punto de vista histológico por la presencia de células malignas mesenquimales productoras de osteide o hueso inmaduro tumoral. A diferencia de otros sarcomas, el osteosarcoma no presenta ninguna alteración molecular o translocación específica. El osteosarcoma convencional (intramedular de alto grado) es el tipo más frecuente y comprende cerca del 90% de todos los casos de osteosarcoma. Según el componente celular predominante en la matriz extracelular, los osteosarcomas se pueden clasificar principalmente en osteoblásticos, condroblásticos o fibroblásticos. Sin embargo, esta clasificación no tiene correlación pronóstica y el manejo médico es idéntico para todos los casos de osteosarcoma de alto grado, independientemente del subtipo histológico [4, 5]. La única excepción es el osteosarcoma central de bajo grado y el osteosarcoma de superficie (paraostal, perióstico y yuxtacortical), que son variantes de bajo grado de malignidad con riesgo de recaída local pero limitado riesgo de diseminación a distancia y que se tratan únicamente con cirugía local con excelente pronóstico [6].

1.2.3. Forma de presentación

La forma de presentación más frecuente del osteosarcoma, es la aparición de dolor del miembro afecto de varias semanas/meses de evolución, inicialmente atribuidos a molestias inespecíficas del crecimiento por la edad de los pacientes, acompañada o no de tumefacción y/o limitación funcional. Aunque poco frecuente, hasta un 10-15% de los pacientes pueden presentar una fractura patológica en el momento del diagnóstico. A diferencia de otros sarcomas de la infancia y adolescencia, la aparición de síntomas sistémicos como fiebre y pérdida ponderal es poco frecuente [5, 7, 8-14].

1.2.4. Etiopatiología

La mayoría de los casos de osteosarcoma son esporádicos, aunque parece existir una asociación con el crecimiento óseo acelerado como demuestra un mayor pico de incidencia cercano al estirón puberal y aparición en zonas metafisiarias, donde se produce el crecimiento óseo (principalmente en la metáfisis de los huesos largos como fémur distal y tibia proxial, seguido de fémur proximal y húmero proximal). No obstante, el osteosarcoma se puede asociar a diversos trastornos genéticos como el retinoblastoma hereditario y los síndromes de Li-Fraumeni, Rothmund –Thomson, Bloom y Werner, por lo que es fundamental una correcta anamnesis incluyendo la historia familiar de cáncer en todos los pacientes.

1.2.5. Diagnóstico y estudio de extensión

La radiología simple del hueso afecto muestra habitualmente características radiológicas de malignidad, como una lesión de crecimiento rápido, mal delimitada, que rompe la cortical. Tanto la tomografía computarizada (TC) como la resonancia magnética (RM), permiten definir mejor las características de la lesión tumoral, aunque únicamente apoyan el diagnóstico de sospecha de malignidad y es, finalmente, la biopsia de lesión tumoral la que permite alcanzar el diagnóstico definitivo. Una vez confirmado el diagnóstico, es fundamental un correcto estudio de extensión a nivel principalmente pulmonar (TC pulmonar) y óseo (gammagrafía ósea o PET-TC), con importante influencia en el tratamiento y en el pronóstico.

1.2.6. Factores pronósticos

Existen distintos factores pronósticos asociados a la supervivencia del osteosarcoma de alto grado [7-14, 34, 35]:

- Al diagnóstico:
    - Presencia de metástasis: los pacientes con metástasis en el momento del diagnóstico inicial, presentan peor pronóstico que pacientes sin evidencia radiológica de diseminación: 24% vs 66%.
    - Localización del tumor primario: los pacientes con tumores de localización axial, presentan peor pronóstico respecto a los pacientes con tumores localizados en extremidades: 27% vs 62%.

- Tamaño del tumor primario: los pacientes con tumores de mayor tamaño (≥10 cm), presentan peor pronóstico que los pacientes con tumores de menor tamaño (< 5 cm): 48% vs 69%.

- Respuesta a la quimioterapia:

  - Grado de necrosis tumoral del tumor primario a la quimioterapia neoadyuvante: los pacientes con tumores con < 90% necrosis tumoral (malos respondedores), tienen peor pronóstico que los pacientes con tumores con ≥ 90% necrosis tumoral (buenos respondedores): 57% vs 80%.

- Resección del tumor primario y/o metástasis:

  - Resección completa macroscópica del tumor primario: Los pacientes con osteosarcoma localizado en los que no se puede realizar cirugía completa, presentan peor pronóstico frente a aquellos en los que sí se puede lograr: 15% vs 65%.

  - Resección completa macroscópica del tumor primario y metástasis: Los pacientes con tumor irresecable, presentan cinco veces más riesgo de éxitus que los pacientes en los que se puede realizar una resección macroscópicamente completa de todas las lesiones tumorales (*Hazard Ratio*: 4.9, intervalo de confianza (IC) 95%: 3.3-7.3).

1.2.7. Tratamiento actual

En el momento del diagnóstico, aproximadamente un 10-20% de los pacientes presentan metástasis detectables con las pruebas de imagen actuales, principalmente pulmonares, con un pronóstico claramente más desfavorable que el resto. Sin embargo, hasta un 60-70% de los pacientes con enfermedad localizada, presentan metástasis pulmonares subclínicas, por lo que es fundamental un tratamiento sistémico, aún en los casos en los que no se haya podido objetivar diseminación a distancia.

Previo a los años 70, el tratamiento del osteosarcoma era exclusivamente quirúrgico. En esa época, a pesar del tratamiento local, el 80% de los pacientes con enfermedad localizada desarrollaba metástasis pulmonares tras la cirugía y fallecían. Como recogía, en

esa época, Sir Stanford Cade: ”If you do not operate they die, if you do operate they die just the same, this meeting should be concluded with prayers”. Gracias a la introducción de la quimioterapia neoadyuvante/adyuvante en los años 70-80, la probabilidad de supervivencia se ha incrementado considerablemente [4, 5]. Sin embargo, desde entonces, no se han producido prácticamente cambios en el tratamiento ni en el pronóstico de estos pacientes (Figura 2) [15].

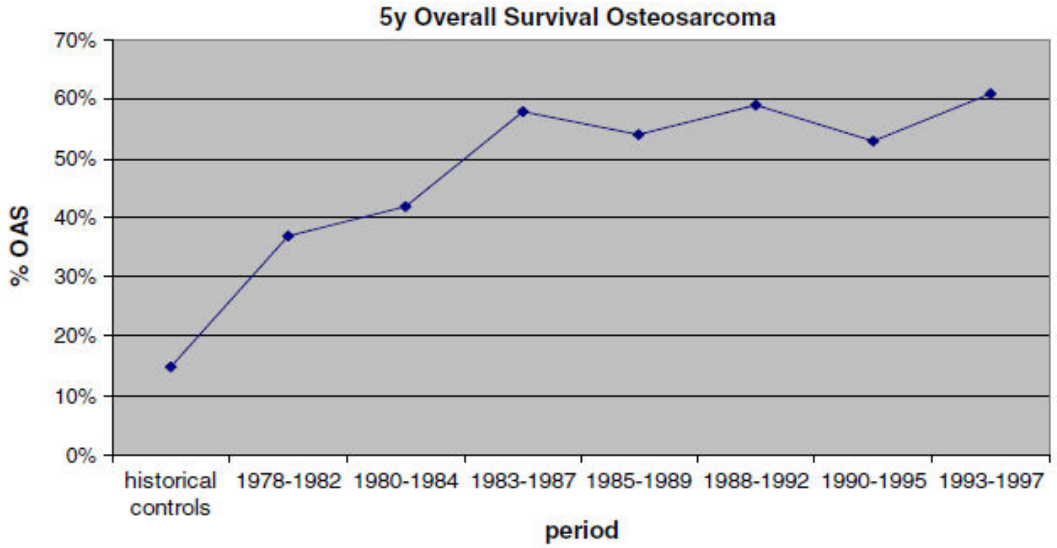

Figura 2: Supervivencia a los 5 años de pacientes con osteosarcoma por período de tratamiento [15]

En la actualidad el tratamiento consiste en quimioterapia neoadyuvante o prequirúrgica seguida de cirugía completa, con bordes libres, del tumor primario y de las metástasis y quimioterapia adyuvante o postquirúrgica. Esto ha permitido alcanzar un 60-70% de supervivencia a los 5 años en los casos de enfermedad inicialmente localizada [7,8]. En los casos con metástasis al diagnóstico, la supervivencia a los 5 años es únicamente del 20-30% [9,10].

Los fármacos quimioterápicos principales en el tratamiento del osteosarcoma son: metotrexato, doxorrubicina, cisplatino, ifosfamida y etopósido. El esquema quimioterápico neoadyuvante y adyuvante de primera línea más utilizado es el esquema MAP (Metotrexato, doxorrubicina/Adriamicina y cisPlatino) [15-20]. Aunque todavía algunos grupos disienten, parece que la adición de ifosfamida a altas dosis ± etopósido al tratamiento neoadjunvante según el esquema MAP no mejora el pronóstico [20-23]. Como ha demostrado recientemente el ensayo clínico fase III aleatorizado EURAMOS-1[24], la adición de Ifosfamida y Etopósido (MAPIE) al tratamiento adyuvante de los pacientes con

bajo grado de necrosis tumoral ("malos respondedores") tratados según el esquema MAP no está indicada, ya que no mejora el pronóstico y aumenta la toxicidad del tratamiento y el riesgo de segundos tumores [16,17, 20, 24].

1.2.8. Nuevos tratamientos

El único fármaco introducido en el tratamiento del osteosarcoma en las últimas décadas es la mifamurtida. La mifamurtida es un fármaco inmunomodulador que actúa activando los monocitos y macrófagos a nivel pulmonar y, potencialmente, disminuye el riesgo de desarrollo de metástasis pulmonares en aquellos pacientes con enfermedad localizada. El ensayo clínico fase III aleatorizado (INT-0133) incluyó 793 pacientes con osteosarcoma localizado $\leq$ 30 años de edad entre 1993 y 1997. Mediante un diseño factorial 2 x 2 se valoró la adición de ifosfamida y/o mifamurtida a la quimioterapia convencional del esquema MAP. La suma de mifamurtida mejoró la supervivencia global, aunque no el riesgo de recaída, mientras que la adición de ifosfamida no mejoró ninguna [23, 24]. Debido a un efecto de interacción entre mifamurtida e ifosfamida, sólo se observó un beneficio en el grupo de pacientes que recibieron ifosfamida concomitante. Por todo ello, aunque la mifamurtida está aprobada en el tratamiento adyuvante del osteosarcoma localizdao por la EMA (Agencia Europea del Medicamento), no ha sido aprobada por su homóloga norteamericana (FDA) y el beneficio real de la mifamurtida continúa siendo fuente de controversia [25].

Asimismo, otros nuevos fármacos con interesantes resultados preclínicos, han sido evaluados en el tratamiento del osteosarcoma, con escasos resultados clínicos:

- Interferón α-2b: en el ensayo clínico fase III aleatorizado EURAMOS-1, la adición de interferón α-2b a la quimioterapia adyuvante de los pacientes con elevado grado de necrosis tumoral, no mejoró el pronóstico [26].

- Trastuzumab: un ensayo clínico fase II con trastuzumab, un anticuerpo monoclonal humanizado frente a HER2, factor pronóstico negativo en el osteosarcoma, no demostró beneficio de la adición de trastuzumab a los pacientes con osteosarcoma metastásico con sobreexpresión de HER2 [27].

- Sorafenib: otro ensayo clínico fase II en pacientes con osteosarcoma irresecable o en recaída con sorafenib, un inhibidor de las tirosina quinasas con efecto

antiangiogénico, demostró eficacia aunque de duración limitada en este grupo de pacientes atribuido a la activación compensadora de la vía de mTOR [28]. Un segundo ensayo clínico fase II probó la adición de everolimus (inhibidor de mTOR) a sorafenib, aunque con escasa actividad [29].

- Ácido zoledrónico: un ensayo clínico fase III en pacientes de nuevo diagnóstico de osteosarcoma aleatorizado con ácido zoledrónico (OS2006), un bifosfonato con prometedores efectos antiangiogénicos e inmunológicos preclínicos, fue suspendido de forma prematura por falta de beneficio [30].

En la actualidad, existen diversos ensayos clínicos con nuevos fármacos diana en fase de reclutamiento en pacientes con osteosarcoma. Algunos de los más interesantes son los que actúan a nivel de las proteínas llamadas de control o "checkpoints", como PD-1 y su ligando PD-L1, que controlan la respuesta de las células inmunitarias frente a los tumores. La expresión de PD-L1 es un factor pronóstico negativo en osteosarcoma [31] y existen diversos ensayos clínicos actualmente abiertos con ipilimumab (anti-CLTA-4), nivolumab/pembrolizumab (anti-PD-1) y atezolizumab (anti-PDL1). Otro de los tratamientos potenciales son los anticuerpos frente a GD2, que es un gangliósido de la superficie de las células del osteosarcoma [32], además de otros tumores. En la actualidad, los anticuerpos antiGD2 son parte del tratamiento de primera línea de los pacientes con neuroblastoma de alto riesgo [33] y su utilidad en pacientes con osteosarcoma está siendo investigada.

Otros nuevos fármacos que están siendo investigados en ensayos clínicos en pacientes con osteosarcoma son: denosumab (anticuerpo monoclonal anti-RANKL), glembatumumab (anticuerpo monoclonal anti-gnNMB) y eribulin (inhibidor de los microtúbulos).

1.3. Situación del cáncer en la infancia y adolescencia en España

1.3.1. Cáncer en la infancia

En España se diagnostican anualmente 850-900 pacientes menores de 15 años afectos de cáncer. Gracias al Registro Español de Tumores Infantiles (RETI-SEHOP), iniciado en los años 80 y de base hospitalaria, sabemos que, al igual que en el resto de países de nuestro entorno, se ha producido un aumento significativo en la supervivencia del cáncer infantil en las últimas décadas (Figura 3) [36, 37].

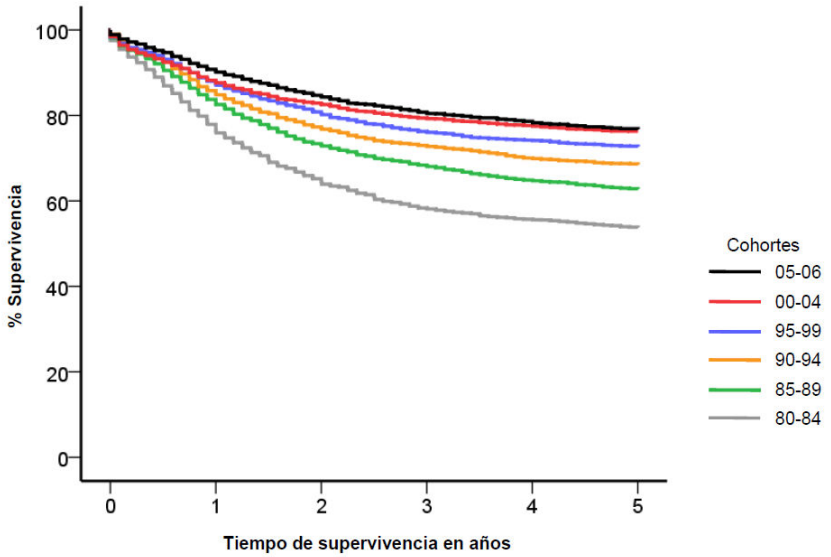

Figura 3: Supervivencia a 5 años del diagnóstico por cohortes de año de incidencia (Pacientes 0-14 años, tumores malignos, Período: 1986-2006, n = 14.965 casos)[37]

1.3.2. El cáncer en la adolescencia

A diferencia de la población infantil, la cobertura del RETI-SEHOP para mayores de 15 años es muy escasa. Las Unidades de Oncología Pediátrica españolas son centros informantes del RETI-SEHOP, con una cobertura mayor del 90-95% de los casos en pacientes < 15 años. Sin embargo, la mayor parte de pacientes mayores de 15 años se tratan en Servicios de Oncología Médica, y éstos no facilitan sus datos a este registro. Por ello, únicamente se dispone de estimaciones de incidencia y supervivencia en este grupo de edad en nuestro país.

Según datos de otros países, la mejoría de supervivencia de los pacientes diagnosticados con cáncer, es menor en el grupo de los adolescentes. Esto se debe a diversos motivos: distinta biología de los tumores, retraso en el diagnóstico, menor tasa de inclusión en ensayos clínicos y mayor dispersión del tratamiento, principalmente [38].

1.3.3. Osteosarcoma en España

El tratamiento del osteosarcoma en España sigue las guías y recomendaciones internacionales previamente descritas y está organizado desde el año 1995 por la Sociedad Española de Oncología Pediátrica (SEOP) (posteriormente Sociedad Española de Oncología y Hematología Pediátricas (SEHOP)). Desde esa fecha, los pacientes con osteosarcoma localizado, se han tratado según las recomendaciones específicas de los protocolos SEOP-SO-95, SEOP-SO-2001, SEHOP-SO-2010 y ISG-GEIS-OS-2. Este último protocolo, actualmente en activo, reúne por primera vez a los grupos de oncología pediátrica y oncología médica españoles unidos en el Grupo Español de Investigación en Sarcomas (GEIS). Los pacientes con osteosarcoma metastásico se trataron, inicialmente, según el protocolo SEOP-SO-95 y, posteriormente, según los protocolos específicos SEOP-SO-MP-2000 y OS-M-SEOP-2011. De todos los protocolos de tratamiento previos, únicamente se han publicado datos del protocolo SEOP-SO-1995 [39], con supervivencia, a los 5 años, similar a las descritas por otros grupos internacionales.

Al igual que ocurrió en el resto de países, gracias a la introducción de la quimioterapia en los años 70-80, se produjo una importante mejoría de supervivencia. Sin embargo, desde entonces, tampoco se han producido prácticamente cambios en el tratamiento ni en el pronóstico de los pacientes con osteosarcoma en nuestro país (Figura 4) [37]. Por ello, son necesarias nuevas estrategias terapéuticas para intentar mejorar el pronóstico de estos pacientes [40].

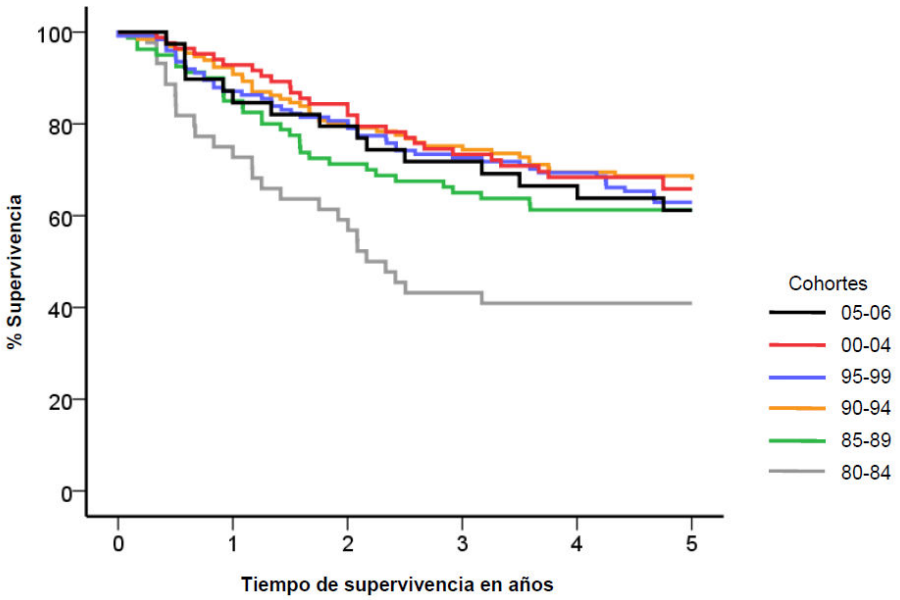

Figura 4: Supervivencia a 5 años del diagnóstico de osteosarcoma por cohortes de año de incidencia (Pacientes 0-14 años, Período: 1986-2006, n = 508 casos) [37]

1.4. Identificación de nuevas dianas terapéuticas en el osteosarcoma

En los últimos años, se postula un nuevo paradigma para el desarrollo de nuevos fármacos antitumorales, basado en la biología tumoral [41, 42]. A diferencia de la quimioterapia convencional, estos nuevos fármacos diana actúan sobre una o varias alteraciones moleculares específicas, identificadas en las células tumorales, pero no en las células normales.

Dentro de las vías de señalización intracelular más importantes, implicadas en el cáncer, destacan las vías de PI3K/AKT/mTOR, RAS/RAF/MEK/ERK y WNT/β-catenina. En la actualidad existen fármacos disponibles en la clínica y en desarrollo de inhibidores específicos de PI3K, AKT o mTORC1/mTORC2, así como de RAF y de MEK.

La activación de la vía de PI3K/AKT/mTOR, se puede producir por distintas alteraciones de la vía, como la activación constitutiva de los receptores tironsinaquinasas, amplificación/mutación de PIK3CA, de AKT o la pérdida de expresión de PTEN. Todas ellas producen una activación constitutiva de la vía que resulta en la fosforilación de AKT (Figura 5) [43]. La activación de AKT promueve resistencia adquirida frente a diversos tratamientos antineoplásicos [44] y se suele asociar a mal pronóstico y resistencia a diversos tratamientos antitumorales [45-48] La activación de la vía de RAS/RAF/MEK/ERK, también se puede producir por mutaciones activantes de receptores tirosina quinasas o por alteraciones de los componentes intracelulares de la vía que confluyen en la fosforilación de ERK (Figura 5) [43, 49]. La vía de WNT/β-catenina controla los niveles intracelulares de β-catenina, de forma que, en ausencia de factores de WNT, los niveles de β-catenina en el citoplasma son bajos. WNT estabiliza la proteína, conllevando el aumento de los niveles citoplasmáticos y su paso al núcleo, en donde se une a los factores de transcripción LEF1 y TCF y permite la transcripción de diversos genes diana [50]. Alteraciones en las vías de PI3K/AKT/mTOR, RAS/RAF/MEK/ERK y WNT/β-catenina son frecuentes en numerosos cánceres, pero existen escasos datos en osteosarcoma [51-54].

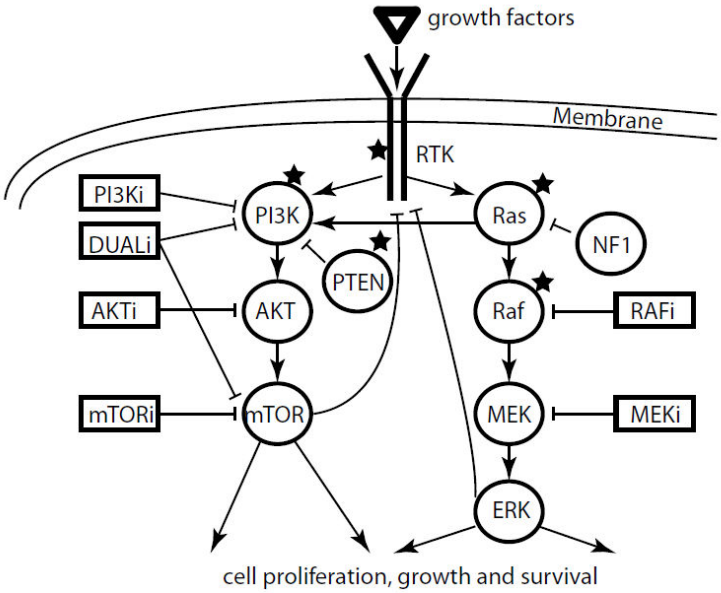

Figura 5: Redes de señales de las vías de PI3K/AKT/mTOR y RAS/RAF/MEK/ERK e inhibidores en desarrollo en ensayos clínicos [43]

Los microRNAs son pequeñas estructuras no codificantes de RNA de 18-25 nucleótidos con actividad post-transcripcional que están implicados en la regulación de la expresión génica. Descubiertos a mediados de los años 90, en los últimos años ha aumentado de forma exponencial el número de publicaciones que describen su participación en distintas funciones fisiológicas y patológicas, como la apoptosis, la proliferación y la diferenciación celular, demostrándose su funcionalidad como genes supresores de tumores o como protoncogenes en la carcinogénesis. Algunos miRNAs están implicados en la regulación de las vías de señalización en las células de osteosarcoma [55].

2. HIPÓTESIS

La identificación de factores pronósticos y nuevas dianas terapéuticas en el osteosarcoma, así como la centralización de su tratamiento puede ayudar a mejorar el pronóstico de estos pacientes.

3. OBJETIVOS

Objetivo general

El objetivo principal de esta tesis es la identificación de factores pronósticos al diagnóstico y en la recaída de los niños y adolescentes con osteosarcoma de alto grado, así como la identificación de nuevas dianas terapéuticas.

Objetivos específicos

Artículo I: Identificación de las características clínico-patológicas, supervivencia global y libre de eventos y factores pronósticos de los pacientes pediátricos y adolescentes con osteosarcoma de alto grado tratados en el Hospital La Fe.

Artículo II: Identificación de las características clínico-patológicas, supervivencia global y libre de eventos y factores pronósticos de los pacientes pediátricos y adolescentes con osteosarcoma localizado de alto grado, en primera recaída, tratados en el Hospital La Fe.

Artículo III: Análisis de la incidencia y distribución del cáncer en la infancia y adolescencia en la Comunidad Valencia. Identificación del lugar y especialista responsable.

Artículo IV: Identificación de nuevas dianas terapéuticas con potencial aplicación clínica en niños y adolescentes con osteosarcoma.

4. JUSTIFICACIÓN DE LA TESIS COMO COMPENDIO DE PUBLICACIONES Y ALCANCE DE LOS RESULTADOS

El principal alcance de los resultados recogidos en las publicaciones incluidas en esta tesis doctoral se detalla a continuación:

Identificación de las características clínico-patológicas, supervivencia global y libre de eventos y factores pronósticos de pacientes pediátricos y adolescentes con osteosarcoma de alto grado al diagnóstico.

Numerosos estudios internacionales han identificado diversos factores pronósticos al diagnóstico que permiten conocer la probabilidad de recaída y supervivencia de pacientes con osteosarcoma. En España, únicamente existen datos publicados de pacientes con osteosarcoma localizado de extremidades tratados entre 1995-2000 según el protocolo SEOP-SO-95. Nuestra publicación supone la primera revisión retrospectiva de más de 25 años de experiencia en el diagnóstico y tratamiento de pacientes pediátricos y adolescentes con osteosarcoma en un centro de referencia nacional, permitiendo identificar factores pronósticos que permitan mejorar el tratamiento y supervivencia de los pacientes con osteosarcoma en nuestro país.

Identificación de las características clínico-patológicas, supervivencia global y libre de eventos y factores pronósticos de pacientes pediátricos y adolescentes con osteosarcoma localizado de alto grado en primera recaída.

A pesar del tratamiento con quimioterapia neoadyuvante, cirugía del tumor primario y quimioterapia adyuvante, aproximadamente el 30-35% de los pacientes con osteosarcoma localizado recaen, con una supervivencia posterior de sólo el 15-35%. Nuestro artículo supone la primera publicación, en nuestro país, del manejo y evolución de pacientes pediátricos y adolescentes con osteosarcoma en primera recaída, con el objetivo de identificar factores que influyan en la supervivencia posterior de este grupo de pacientes.

Análisis de la incidencia y distribución del cáncer en la infancia y adolescencia. Dispersión del tratamiento.

El osteosarcoma tiene su mayor incidencia en la adolescencia. En las últimas décadas se ha producido un incremento en la supervivencia de los pacientes con cáncer, sin embargo esta mejoría ha sido menor en el grupo de adolescentes. Uno de los principales factores identificados es la mayor dispersión del tratamiento de estos pacientes respecto a la población infantil. En esta publicación analizamos, a partir de los datos del Registro de Tumores Infantiles de la Comunidad Valenciana, la incidencia y lugar de tratamiento de los adolescentes en nuestro país. Este análisis permite, por primera vez, conocer la situación real del tratamiento de los adolescentes adolescentes con cáncer en nuestro medio.

Identificación de nuevas dianas terapéuticas con potencial aplicación clínica en niños y adolescentes con osteosarcoma.

El osteosarcoma es uno de los tumores de peor pronóstico en la infancia y adolescencia. A pesar de la mejoría de la supervivencia inicial tras la introducción de la quimioterapia neoadyuvante/adyuvante, no se han producido cambios en el pronóstico de estos pacientes en las últimas décadas. En caso de recaída o progresión, principalmente a nivel pulmonar, las posibilidades de curación son bajas. En esta publicación analizamos los mecanismos moleculares implicados en la progresión metastásica del osteosarcoma, con potencial aplicación terapéutica.

5. RESULTADOS Y DISCUSIÓN

Identificación de las características clínico-patológicas, supervivencia global y libre de eventos y factores pronósticos de pacientes pediátricos y adolescentes con osteosarcoma de alto grado al diagnóstico.

Resultados:

Se han analizado 77 pacientes ≤ 21 años con osteosarcoma de alto grado diagnosticados y tratados en la Unidad de Oncología Pediátrica y el Servicio de Oncología Médica del Hospital La Fe de Valencia, entre Enero 1985 y Diciembre 2011. Las características clínicas y biológicas se detallan en Tabla 1, Artículo I. La supervivencia libre de eventos (SLE) es de 38 ± 11% a los 5 y 10 años; la supervivencia global (SG) de 51 ±12% y 45 ± 12% a los 5 y 10 años respectivamente (Figura 1, Artículo I), con una mediana de seguimiento de 11 años (rango: 1.6-26.4 años).

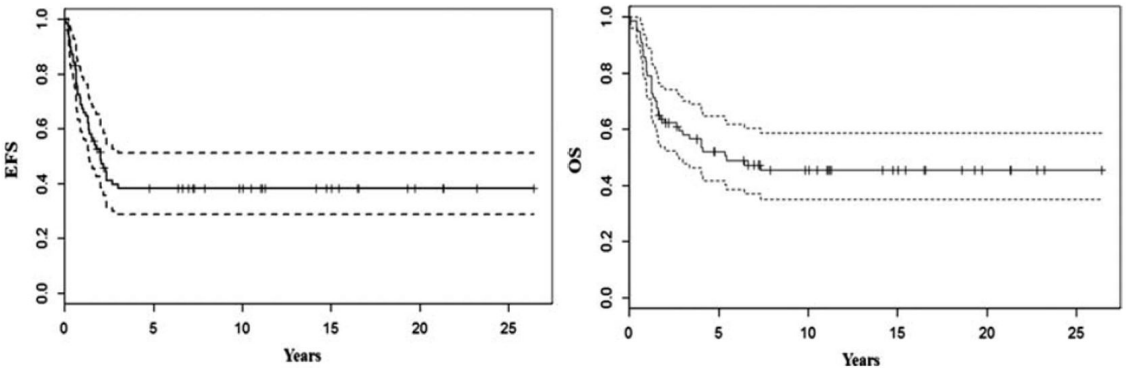

Figura 1, Artículo I: SLE (EFS) y SG (OS) de pacientes con osteosarcoma (IC 95%)

La SLE y SG a 5 años es claramente inferior en los pacientes con enfermedad diseminada al diagnóstico (10% ± 10%) comparada con los pacientes con enfermedad localizada (62 ± 12% y 57 ± 12% respectivamente, Figura 2, Artículo I).

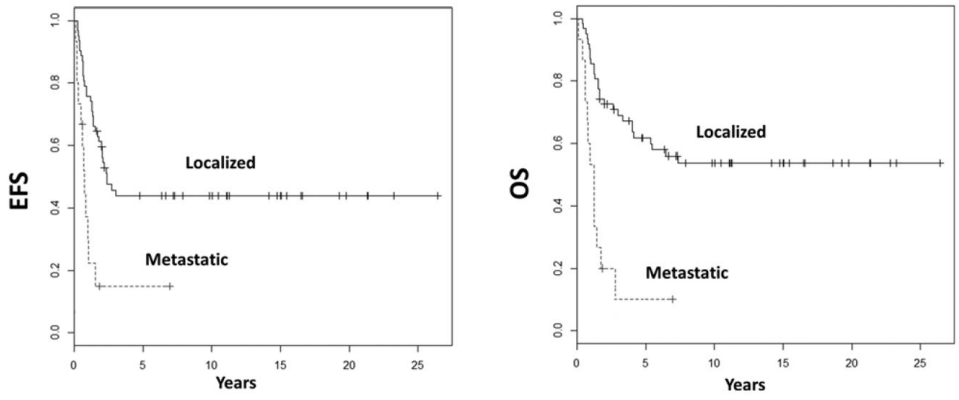

Figura 2, Artículo I: SLE (EFS) y SG (OS) en función de diseminación de la enfermedad

En el análisis univariante de factores pronósticos de supervivencia únicamente la presencia de metástasis al diagnóstico, el bajo grado de necrosis tumoral como respuesta a la quimioterapia neoadyuvante, el retraso en el reinicio de la quimioterapia adyuvante tras la cirugía del tumor primario y el no alcanzar una respuesta completa (RC) con el tratamiento de primera línea, se asociaron con peor supervivencia (Tabla 2, Artículo I).

Variable	Survivors (n = 37)	Exitus (n = 40)	*P*
Age	13.9 ± 3.7	12.7 ± 4.6	0.201
Sex (n [%])			0.180
Male	22 (59.5)	20 (50)	
Female	15 (40.5)	20 (50)	
Primary metastatic disease (n [%])			0.000
No	35 (94)	27 (68)	
Yes	2 (6)	13 (32)	
Site primary disease (n [%])			0.089
Extremity	36 (97.3)	35 (87.5)	
Axial	1 (2.7)	5 (12.5)	
Symptomatic time to diagnosis (wk)	10 (1-70)	8 (1-100)	0.435
Treatment Unit (n [%])			0.951
Pediatric Oncology	22 (59.5)	21 (52.5)	
Medical Oncology	15 (40.5)	19 (47.5)	
Time CTX-Sx (d)	24 (11-46)	24 (9-72)	0.146
Time Sx-CTX (d)	26 (15-62)	34.5 (14-122)	0.082
Time CTX-Sx-CTX (d)	50 (26-77)	63 (26-139)	0.021
Surgery (n [%])			0.203
Conservative	29 (78.4)	30 (75)	
Radical	8 (21.6)	10 (25)	
Week of surgery	14 (0-28)	13 (0-35)	0.724
Tumor necrosis rate (%)	80 (10-100)	30 (0-100)	0.004
RC at the end of first-line treatment (n [%])	37 (100)	15 (37.5)	0.000
Period of treatment (n [%])			
≤1997	19 (51.4)	21 (52.5)	0.892
> 1997	18 (48.6)	19 (47.5)	

Tabla 2, Artículo I: Análisis univariante de factores pronósticos

En el análisis multivariante, únicamente el bajo grado de necrosis tumoral y la ausencia de RC con el tratamiento de primera línea, se asociaron con peor pronóstico (Tabla 3, Artículo I).

Variable	Hazard Ratio	95% CI	*P*
Primary metastatic disease	3.047	0.817-11.357	0.097
Time CTX-Sx-CTX	0.999	0.979-1.019	0.913
Tumor necrosis rate	0.982	0.967-0.998	0.026
RC end first-line treatment	50.004	8.764-285.307	< 0.001

Tabla 3, Artículo I: Análisis multivariante de factores pronósticos

Discusión:

Las características clínicas y biológicas de nuestros pacientes son similares a las previamente descritas en la literatura. A pesar del amplio periodo analizado (1985-2011), el tratamiento del osteosarcoma no se ha modificado desde principios de los años 80 y todos los pacientes recibieron al menos 3 de los 4 quimioterápicos de eficacia reconocida en el tratamiento del osteosarcoma (doxorrubicina, metotrexato, cisplatino e ifosfamida). El osteosarcoma se localiza principalmente a nivel de las extremidades y un 19% de los pacientes de nuestra serie debutaron con metástasis, principalmente pulmonares. En nuestra serie destaca el escaso porcentaje de pacientes con buena respuesta a la quimioterapia prequirúrgica (32‰ vs. 50-60% descrito en la literatura) independientemente de los diferentes protocolos de quimioterapia empleados. Otra diferencia respecto a otras series es que el tiempo desde la cirugía del tumor primario hasta el reinicio de la quimioterapia adyuvante, factor pronóstico reconocido, fue en nuestros pacientes mayor de las 3-4 semanas recomendadas, sin justificación aparente.

Los pacientes con metástasis pulmonares al diagnóstico presentan peor pronóstico que los pacientes con enfermedad localizada. Sin embargo, no existe consenso en lo relativo a la definición de metástasis pulmonares. La definición más ampliamente utilizada de metástasis pulmonares es: "un nódulo pulmonar único $\geq$ 10 mm o $\geq$ 3 nódulos pulmonares $\geq$ 5mm". Sin embargo diversas publicaciones consideran metástasis pulmonar a "cualquier nódulo pulmonar > 5 mm" o "cualquier nódulo pulmonar independientemente del tamaño" y otras, ni siquiera lo especifican. Por el contrario, en otras publicaciones restringen esta definición a exclusivamente a aquellas "metástasis comprobadas por cirugía o progresión". La evolución de las técnicas de imagen como la TC permite la identificación de pequeños nódulos pulmonares que previamente hubieran pasado inadvertidos. En nuestro caso, de los 9 pacientes con lesiones pulmonares al diagnóstico que, por criterios de número y tamaño número, no hubieran sido consideradas metástasis, cinco fueron considerados metástasis a posteriori. Por ello, la mejoría en la calidad de la imagen del TC ha permitido incrementar la sensibilidad de detección de nódulos pulmonares y, potencialmente, podría mejorar la supervivencia de los pacientes con osteosarcoma al permitir un manejo más agresivo de dichos nódulos pulmonares.

Identificación de las características clínico-patológicas, supervivencia global y libre de eventos y factores pronósticos de pacientes pediátricos y adolescentes con osteosarcoma localizado de alto grado en primera recaída.

Resultados:

Se han analizado 31 pacientes ≤ 21 años en primera recaída de un osteosarcoma de alto grado inicialmente localizado, diagnosticados y tratados en la Unidad de Oncología Pediátrica y el Servicio de Oncología Médica del Hospital La Fe de Valencia, entre Enero 1985 y Diciembre 2011. Las características clínicas y biológicas de los pacientes en el momento del diagnóstico inicial y de la primera recaída se detallan en Tabla 1. La mediana de tiempo, desde el diagnóstico hasta la recaída, fue de 16 meses (rango: 3-36 meses). El tipo de recaída principal fue pulmonar y el tratamiento principal fue quimioterapia y cirugía. De los pacientes que alcanzaron una segunda respuesta completa, un tercio son supervivientes a largo plazo (Figura 1, Artículo II). La SG y SLE a los 5 años tras la recaída fue del 26% (IC95%: 14-49%) (Figura 2, Artículo II), con una mediana de seguimiento de 99 meses (rango: 27-271 meses).

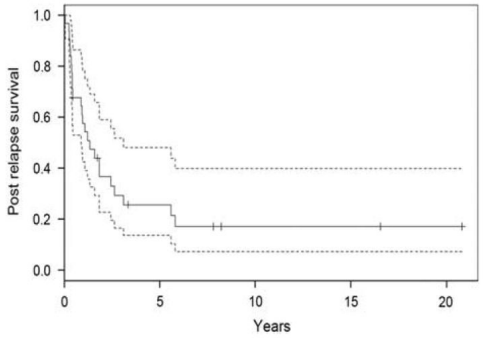

Figura 2, Artículo II: SG post recaída de pacientes con osteosarcoma localizado (IC 95%)

En el análisis univariante, únicamente el tiempo desde el diagnóstico inicial hasta la primera recaída y como no alcanzar una segunda respuesta completa con el tratamiento de la recaída, se asociaron con el pronóstico (Tabla 2, Artículo II; Figura 3, Articulo II).

Variables	Survivors (n = 7)	Exitus (n = 24)	*P*
Age at diagnosis (y)	11.5 (10.0-21.0)	14.1 (1.9-20.6)	0.73
Sex			
Male	1 (14)	14 (58)	0.12
Female	6 (86)	10 (42)	
Site of relapse			
Lung/bone only	4 (57)	13 (54)	0.32
Local only	2 (29)	5 (21)	
Combined relapse	1 (14)	6 (25)	
Time to first relapse (mo)	24 (16-36)	11 (3-32)	< 0.001
Histologic response (first CR)	70 (20-95)	40 (0-100)	0.10
Relapse during treatment	0	11 (46)	< 0.001
Lung involvement			0.11
Unilateral	3 (75)	5 (29)	
Bilateral	1 (25)	12 (71)	
No. lung metastases			0.23
1	2 (50)	3 (18)	
2	1 (25)	2 (12)	
≥ 3	1 (25)	12 (70)	
Surgery at first relapse	7 (100)	14 (58)	< 0.001
Chemotherapy at first relapse	5 (71)	19 (80)	0.91
Achieved second CR	6 (86)	8 (33)	< 0.001

Tabla 2, Artículo II: Análisis univariante de factores pronósticos

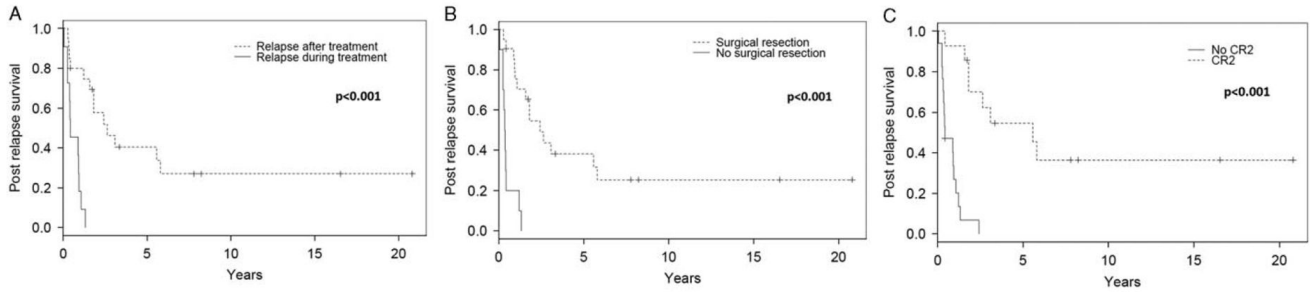

Figura 3, Artículo II: SG post recaída: A) RC1, B) Cirugía de la recaída, C) CR2

En el análisis multivariante, únicamente el grado de necrosis tumoral a la quimioterapia de primera línea, así como alcanzar una segunda respuesta completa, se asoció con el pronóstico (Tabla 3, Artículo II).

Variables	Hazard Ratio	95% CI	*P*
Achievement of second CR	0.016	0.002-0.156	< 0.001
Histologic response	0.976	0.956-0.996	0.020
Time to first relapse	1.001	0.934-1.090	0.821

Tabla 3, Artículo II: Análisis multivariante de factores pronósticos

Discusión:

Las características de nuestros pacientes al diagnóstico inicial y en la primera recaída, así como la SLE y SG a los 5 años tras la recaída, son similares a las descritas previamente. Como excepción, en nuestra serie hay una mayor proporción de recaídas locales aisladas (23%) o combinadas (13%), comparadas con otras series (5-22% y 3-8%, respectivamente). Todo ello probablemente en relación con el escaso porcentaje de pacientes de nuestra serie (15%) que presentaron buena respuesta a la quimioterapia de primera línea (definida como grado de necrosis tumoral ≥ 90%, uno de los factores principales asociados al riesgo de recaída local), frente al 27-56% descrito en otras series. Asimismo, la mayoría de las recaídas locales se produjeron en el periodo 1985-1995, por lo que una mejora en la calidad de la cirugía local puede tener igualmente influencia.

Respecto a los factores pronósticos en la primera recaída, la obtención de una segunda respuesta completa mediante resección quirúrgica de todas las lesiones fue el factor pronóstico más importante. En nuestra serie, de los catorce pacientes en los que se alcanzó una segunda respuesta quirúrgica completa, seis son supervivientes a largo plazo.

El grado de necrosis tumoral a la quimioterapia de primera línea, así como el tiempo desde el diagnóstico inicial hasta la recaída, son factores pronósticos clásicos. Sin embargo, al considerar el grado de necrosis tumoral como variable continua, el tiempo desde el diagnóstico hasta la recaída pierde significación estadística en el análisis multivariante de nuestra serie. Es decir, los pacientes con peor respuesta a la quimioterapia de primera línea recaen de forma más precoz, con un peor pronóstico.

Análisis de la incidencia y distribución del cáncer en la infancia y adolescencia. Dispersión del tratamiento.

Resultados:

El Registro de Tumores Infantiles de la Comunidad Valenciana (RTICV) es un registro de base poblacional que recoge casos de cáncer infantil (0-14 años) desde 1983 y de adolescentes (15-19 años) desde 2007 (Figura 1, Artículo III).

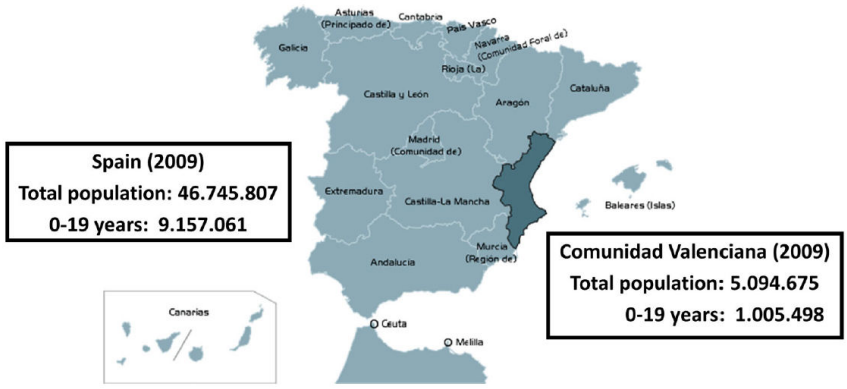

Figura 1, Artículo III: España y la Comunidad Valenciana (Instituto Nacional Estadística)

En el período 2007-2010, se registraron 696 nuevos casos de cáncer en pacientes residentes en la Comunidad Valenciana menores de 20 años, de ellos 183 casos entre 15 y 19 años. La incidencia global ajustada por edad fue de 176.0 casos por millón (IC 95%: 162.8-189.2), con mayor incidencia en el grupo de pacientes menores de un año (287.4), seguidos de 1-4 años (205.5), adolescentes (179.9), 10-14 años (150.2) y 5-9 años (140.6). El grupo de edad de 15-19 años presentó neoplasias intermedias entre la edad pediátrica y la edad adulta, siendo los más frecuentes los linfomas, seguidos de tumores del sistema nervioso central, carcinomas, tumores óseos, leucemias y tumores germinales (Tabla 1, Artículo III).

Diagnostic group (ICCC-3)	0 years old	1–4 years old	5–9 years old	10–14 years old	15–19 years old	ASRw (95 % CI)
I. Leukemias	55.5	76.0	51.3	22.4	27.7	45.7 (38.9–52.5)
II. Lymphomas	10.1	16.6	16.4	29.8	45.5	25.6 (20.7–30.5)
III. CNS neoplasms	40.3	36.8	39.0	33.0	33.6	36.0 (30.1–42.0)
IV. Neuroblastoma	70.6	28.5	2.1	1.1	1.0	12.1 (8.4–15.7)
V. Retinoblastoma	15.1	7.1	1.0	0.0	0.0	2.9 (1.1–4.7)
VI. Renal tumors	20.2	17.8	4.1	1.1	0.0	6.8 (4.0–9.5)
VII. Liver tumors	20.2	3.6	0.0	1.1	1.0	2.5 (0.9–4.2)
VIII. Bone tumors	0.0	3.6	11.3	30.9	19.8	15.1 (11.3–18.8)
IX. Soft tissue sarcoma	25.2	10.7	10.3	7.5	8.9	10.3 (7.1–13.5)
X. Germ cell tumors	15.1	3.6	1.0	9.6	16.8	8.0 (5.2–10.7)
XI. Other carcinomas/melanomas	15.1	1.2	4.1	13.8	25.7	11.1 (7.9–14.3)
Total	287.4	205.5	140.6	150.2	179.9	176.0 (162.8–189.2)

Tabla 1, Artículo III: Incidencia de cáncer por millón en la C. Valenciana 2007-2010

Respecto al lugar de tratamiento, la mayoría de los pacientes < 20 años fueron tratados en unidades de oncología pediátrica, disminuyendo de forma proporcional a la edad (Figura 2, Artículo III).

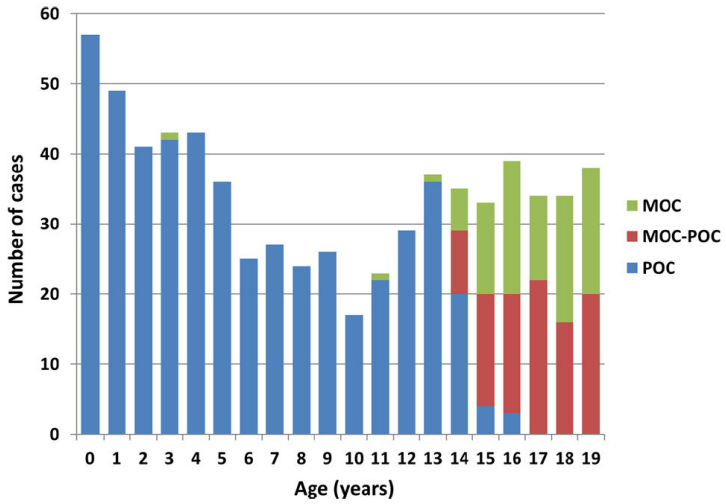

Figura 2, Artículo III: Lugar de tratamiento de los pacientes con cáncer en la Comunidad Valenciana (2007-2010) (POC = Unidad de Oncología Pediátrica, MOC= Servicio de Oncología Médica).

Mientras prácticamente todos los pacientes < 14 años fueron tratados en una de las tres unidades de referencia de oncología pediátricas de la Comunidad Valenciana, el tratamiento de los adolescentes diagnosticados en este período fue mucho más disperso (Tabla 2, Artículo III): el 60% se trataron en uno de los tres hospitales que dispone de unidad de referencia de oncología pediátrica, mientras que el 40% restante fue tratado en un total de 20 centros distintos, con una mediana de un paciente adolescente/año por centro.

	14 years				15–19 years			
	POC	MOC-POC	MOC	Total	POC	MOC-POC	MOC	Total
I. Leukemias	2	0	0	2	5	21	2	28
II. Lymphomas	6	0	1	7	0	22	25	47
III. CNS Neoplasms	4	3	2	9	0	20	14	34
IV. Neuroblastoma	0	0	0	0	0	1	0	1
V. Retinoblastoma	0	0	0	0	0	0	0	0
VI. Renal tumors	0	0	0	0	0	0	0	0
VII. Liver tumors	1	0	0	1	0	1	0	1
VIII. Bone tumors	3	0	3	6	1	16	3	20
IX. Soft tissue sarcoma	2	0	1	3	1	3	5	9
X. Germ cell tumors	2	0	0	2	0	4	13	17
XI. Other carcinomas/melanomas	0	3	2	5	0	8	18	26
Total	20	6	9	35	7	96	80	183

POC Pediatric Oncology Center, *MOC-POC* Medical Oncology Center with a Pediatric Oncology Center within the same institution, *MOC* Medical Oncology Center without a Pediatric Oncology Center within the same institution

Tabla 2, Artículo III: Lugar de tratamiento de los adolescentes en la C. Valenciana (2007-2010)

Discusión:

No existe ningún registro de tumores de adolescentes nacional, por lo que estos datos de RTICV suponen el primer estudio epidemiológico basado en datos poblacionales llevado a cabo en España sobre los adolescentes con cáncer. Estos datos nos permiten conocer, no sólo la incidencia del cáncer en este grupo de edad, sino también el lugar de tratamiento de estos pacientes. Teniendo en cuenta todos los grupos tumorales, la incidencia de cáncer en la adolescencia es mayor que en el grupo de edad de 5-14 años, similar a la descrita en otros países de nuestro entorno. El espectro de neoplasias en la adolescencia es específico e intermedio entre la infancia y la edad adulta, presentando una transición entre las neoplasias de origen embrionario y las neoplasias de origen epitelial.

Diversos consensos nacionales e internacionales recalcan la importancia de un tratamiento especializado de los adolescentes con cáncer, especificando que los pacientes < 19 años "deben de ser tratados en instalaciones adecuadas a su edad", "de acuerdo al mejor protocolo de tratamiento disponible" y "por personal experto en el tipo de tumor y tratamiento ofrecido", así como tener la oportunidad de ser incluidos en los ensayos clínicos para los que sean elegibles" (estándares europeos de atención a los niños con cáncer, guías NICE, etc.). En España, el II Plan Estratégico Nacional de Infancia y Adolescencia (2013-2016), recomienda la ampliación de la edad pediátrica hasta los 18 años para facilitar la centralización del tratamiento de los adolescentes.

Los datos analizados indican una importante y preocupante dispersión del tratamiento de los adolescentes con cáncer en la Comunidad Valenciana, en clara contraposición a la centralización del tratamiento de la población pediátrica. El tratamiento de adolescentes con cáncer en múltiples centros, muchos de ellos sin equipos con experiencia en tumores pediátricos, podría suponer una merma en la calidad asistencial al no contar con los mejores recursos y experiencia (no sólo de oncólogos y cirujanos, sino también patólogos, radiólogos, etc.) que aseguren el mejor tratamiento para este grupo de pacientes. Es fundamental la centralización del tratamiento de este grupo de pacientes en unidades con experiencia reconocida, así como impulsar la colaboración entre los equipos de oncología pediátrica y oncología médica que permitan que los adolescentes puedan recibir el mejor tratamiento en base a la experiencia conjunta de ambos equipos y los ensayos clínicos disponibles. Para ello lo recomendable sería la creación de unidades

específicas para el diagnóstico y tratamiento de los adolescentes y adultos jóvenes con cáncer, fruto de la integración de equipos de oncología pediátrica y oncología médica, como es el caso de otros países europeos.

Identificación de nuevas dianas terapéuticas con potencial aplicación clínica en niños y adolescentes con osteosarcoma (miR-200c y pAKT).

Resultados:

Se han analizado muestras tumorales de 36 pacientes diagnosticados y tratados en el Hospital La Fe entre 1990-2011. Sus características clínico-patológicas están recogidas en la Tabla 1, Artículo IV.

Estudio de inmunohistoquímica: Tras revisión y selección de las muestras de tumor conservada en parafina por el patólogo, se procedió a la elaboración de *Tissue Microarrays* (TMAs). Se analizaron mediante inmunohistoquímica la activación de las rutas de PI3K/Akt/mTOR (AKT-pS473), RAS/RAF/MEK/ERK (p-Erk1,2), WNT/β-catenina (β-catenin) y Sonic-Hedgehog (Gli1). Para su interpretación se utilizó el HSCORE. El único factor pronóstico estadísticamente significativo en el análisis multivariante fue la activación de la vía de PI3K/AKT/mTOR (p-AKT), en las muestras tumorales previas al inicio de la quimioterapia (Figura 1, Artículo IV).

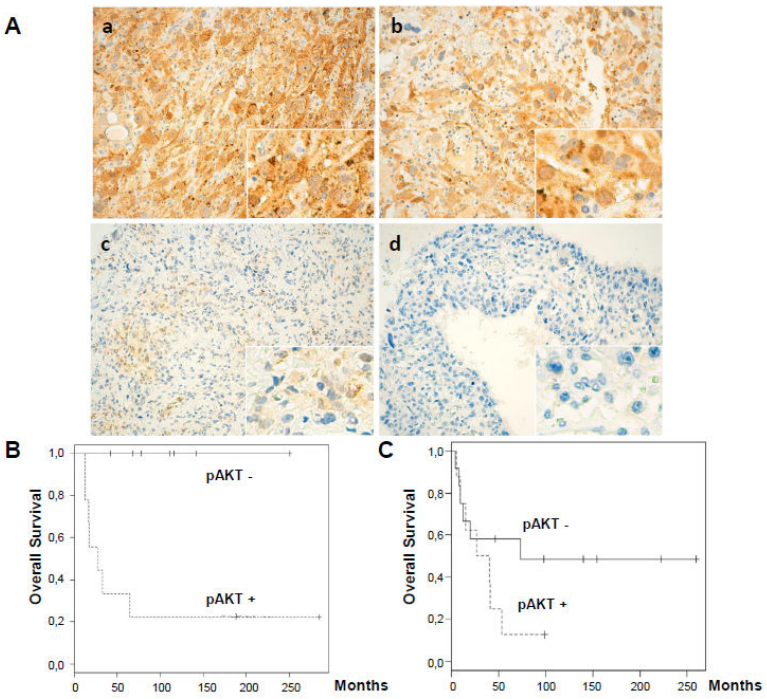

Figura 1, Artículo IV: A) Estudio inmunohistoquímico pAKT, B) Supervivencia global en función de pAKT.

Estudio de miRNAs: Se extrajo RNA a partir de las muestras de tumor seleccionadas por los patólogos. El muestras obtenidas se hibridaron en los *microarrays* Genomic miRNA 3.0 de Affymetrix. Se analizó el perfil de expresión de miRNAs entre

tumores primarios (n=11) y metástasis pulmonares (n=15) mediante regresión logística Elastic Net. Los únicos miRNAs con diferente expresión entre tumores primarios y metástasis pulmonares fueron miR200c, miR4786-3p y ENSG00000212490_X (Figura 2, Artículo IV). Estos tres miRNAs fueron validados en una segunda cohorte independiente de 16 casos de osteosarcoma de características similares procedentes del Hospital Clínico de Valencia, confirmando la diferente expresión de miR200c entre tumores primarios y metástasis pulmonar. Asimismo, la expresión de miR200c se correlacionó, de forma estadísticamente significativa, con la expresión de miR-375 y miR141 (Figura 3, Artículo IV), ambos implicados en la transición mesénquima-epitelio.

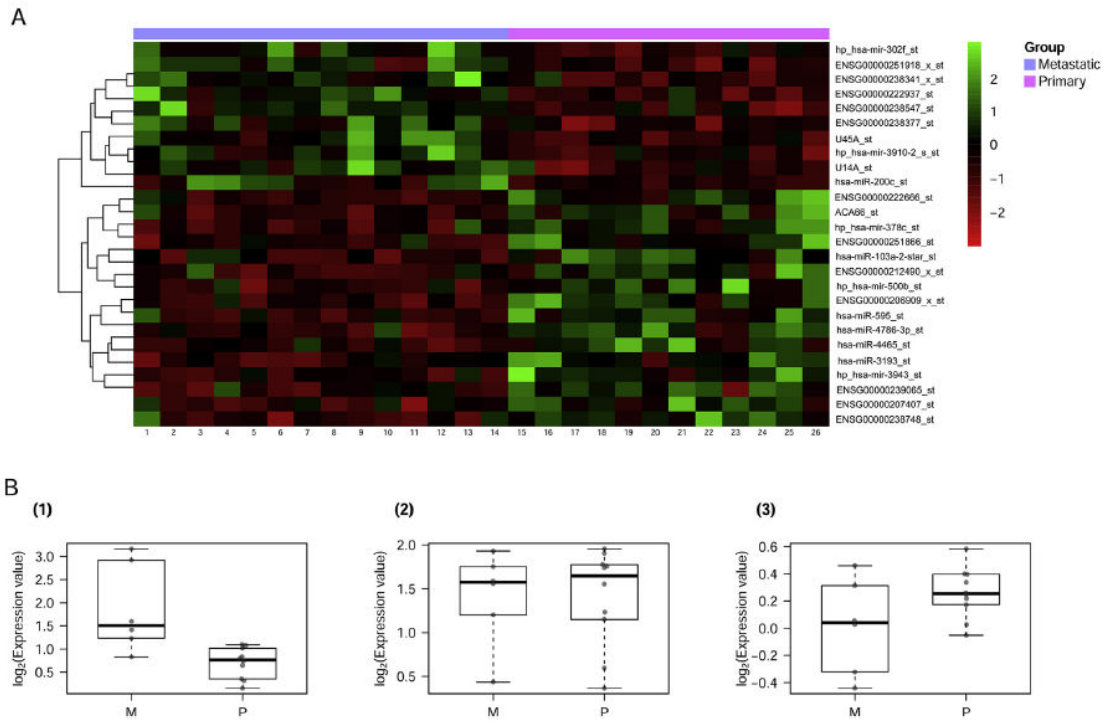

Figura 2, Artículo IV: A) Perfil de expresión de miRNA, B) Cohorte validación: (1) miR200c (p=0.003), (2) miR4786-3p (p=0.96) y (3) ENSG00000212490_X (p=0.23)

Para confirmar el papel de miR200c en el desarrollo de metástasis en el osteosarcoma, se realizó la transfección de miR200c mimic en la línea celular de osteosarcoma U2-OS, observándose un aumento de proliferación celular y migración (Figura 4), así como una disminución de p-AKT (Figura 5, Artículo IV). En base a estos resultados, nuestra hipótesis es que la sobreexpresión de miR200c es uno de los mecanismos responsables del desarrollo de metástasis pulmonares en el osteosarcoma. El análisis in silico, demuestra una mayor expresión de E-cadherina en las metástasis pulmonares frente a los tumores primarios de osteosarcoma, apoyando nuestra hipótesis de

que el osteosarcoma metastatiza a nivel pulmonar mediante la transición mesénquima-epitelio.

Discusión:

Nuestros resultados demuestran que la activación de PI3K/AKT/mTOR determinada mediante pAKT en el tumor primario previo al inicio de la quimioterapia, se asocia a peor pronóstico en los pacientes con osteosarcoma. La inhibición de la vía de PI3K/AKT/mTOR, en combinación con quimioterapia u otras terapias diana, podría ser de interés clínico como recientemente comunicado por el grupo francés de sarcomas [56]. En la actualidad, disponemos en la clínica únicamente de inhibidores de mTOR (sirolimus, everolimus, temsirolimus) que actúan sobre el complejo MTORC1, pero no sobre MTORC2. Diversos inhibidores de PI3K, AKT y MTORC1/2 están actualmente en desarrollo en ensayos clínicos en pacientes adultos.

Nuestros resultados demuestran, asimismo, un nuevo papel de miR-200c, sobreexpresado en las metástasis pulmonares comparadas con el tumor primario, en el desarrollo de metástasis pulmonares en los pacientes con osteosarcoma. Estudios previos implican a miR200c en la transición epitelio-mesenquimal, sin embargo nuestros resultados demuestran que miR200c facilita la diseminación a distancia mediante el proceso de transición mesénquima-epitelio, lo que explicaría por qué el osteosarcoma disemina, principalmente, a pulmón y no a otras localizaciones como médula ósea. En la actualidad, no existen fármacos que actúen directamente sobre miR-200c.

Finalmente y resumiendo los resultados de todos los estudios concluimos que:

Los factores de riesgo y el pronóstico de los pacientes con osteosarcoma de alto grado al diagnóstico y tras la primera recaída en nuestro medio son similares a otros grupos. La resección quirúrgica de todas las lesiones tumorales es imprescindible, ya sea en primera línea o en caso de recidiva de la enfermedad. La activación de la vía de PI3K/AKT/mTOR y la sobreexpresión de miR200c se correlacionan con peor pronóstico y desarrollo de metástasis pulmonares, respectivamente, por lo que su inhibición podría tener potencial aplicación terapéutica. Por último, es fundamental la creación de unidades específicas para el tratamiento de los adolescentes con cáncer, fruto de la colaboración de las unidades de oncología pediátrica y oncología médica, que permita mejorar su tratamiento y su pronóstico.

6. CONCLUSIONES

Osteosarcoma is the most common primary malignant bone tumour in children and adolescents. Although initial improvement of long-term survival in the 1970s and 80s, there has been no further substantial improvement of survival since then.

Regarding the identification of characteristics, prognostic factors and overall and event-free survival of paediatric and adolescent patients with high-grade osteosarcoma at diagnosis, we conclude that:

1. The 5-year OS and EFS of high-grade osteosarcoma in our cohort was 62 ± 12% y 57 ± 12%, respectively.
2. Main prognostic factors at diagnosis were: metastatic disease, poor histologic response to first-line induction chemotherapy and incomplete surgical removal of all detectable lesions.
3. There is a need for a new definition of "pulmonary metastases" at diagnosis that takes into account current imaging techniques improvements and limitations.

With regard to the identification of characteristics, prognostic factors and overall and event-free survival of paediatric and adolescent patients with localized high-grade osteosarcoma at relapse, we conclude that:

1. The 5-year OS and SLE post relapse was 26% (IC95%:14-49%).
2. Main prognostic factors at relapse were: histologic response to neoadjuvant first-line chemotherapy and complete surgical removal of all lesions at relapse.

Regarding the determination of the incidence and cancer distribution, treatment setting and provider specialty of children and adolescents in the Comunidad Valenciana, we conclude that:

1. Adolescents have higher cancer incidence than children 5-14 years old.
2. There is an important dispersion of treatment of adolescents compared to children in the Comunidad Valenciana. We suggest the creation of specific teenager and young adult cancer centres for their treatment.

With regard to the identification of new therapeutic targets in paediatric and adolescent osteosarcoma, we conclude that:

1. PI3K/AKT/mTOR activation, determined by phopho-AKT immunostaining, is associated with lower overall survival in osteosarcoma primary tumours.
2. MiR-200 is overexpressed in lung metastases and plays a role in the molecular processes of lung metastasis.
3. PI3k/AKT/mTOR and miR-200c inhibitors are potential therapeutic targets to prevent progression and metastasis of paediatric osteosarcomas.

7. REFERENCIAS

1. Gatta G, Botta L, Rossi S, Aareleid T, Bielska-Lasota M, Clavel J, Dimitrova N, et al. Childhood cancer survival in Europe 1999-2007: results of EUROCARE-5—a population-based study. Lancet Oncol. 2014; 15: 35-47.
2. Foucher E, Stiller C, Lacour B, Kaatsch P. International Classification of Childhood Cancer, third edition. Cancer. 2005;103:1457–67.
3. Gurney JG SA, Bulterys M. Malignant bone tumors. In: Cancer Incidence and Survival Among Children and Adolescents: United States SEER Program 1975-1995, Ries LA, Smith MAS, Gurney JG, et al (Eds), SEER program, National Cancer Institute, Bethesda MD 1999 p99.
4. Bielack S, Kempf-Bielack B, Von Kalle T, Schwarz R, Wirth T, Kager L, et al. Controversies in childhood osteosarcoma. Minerva Pediatr. 2013; 65: 125-48.
5. Ritter J, Bielack SS. Osteosarcoma. Ann Oncol. 2010; 21: 20-5.
6. Klein MJ, Siegal GP. Osteosarcoma: anatomic and histologic variants. Am J Clin Pathol. 2006;125: 555-81.
7. Bielack S, Jurgens H, Jundt G, Kevric M, Kuhne T, Reichardt P, et al. Osteosarcoma: the COSS experience. Cancer Treat Res. 2009; 152: 289-308.
8. Ferrari S, Palmerini E, Staals EL, Mercuri M, Franco B, Picci P, et al. The treatment of nonmetastatic high grade osteosarcoma of the extremity: review of the Italian Rizzoli experience. Impact on the future. Cancer Treat Res. 2009; 152: 275-87.
9. Mialou V, Philip T, Kalifa C, Perol D, Gentet JC, Marec-Berard P, et al. Metastatic osteosarcoma at diagnosis: prognostic factors and long-term outcome--the French pediatric experience. Cancer. 2005; 104: 1100-9.
10. Kager L, Zoubek A, Potschger U, Kastner U, Flege S, Kempf-Bielack B, et al. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. J Clin Oncol. 2003 15; 21: 2011-8.
11. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol. 2002; 20: 776-90.
12. Whelan JS, Jinks RC, McTiernan A, Sydes MR, Hook JM, Trani L, et al. Survival from high-grade localised extremity osteosarcoma: combined results and prognostic

factors from three European Osteosarcoma Intergroup randomised controlled trials. Ann Oncol. 2012; 23: 1607-16.

13. Picci P, Mercuri M, Ferrari S, et al. Survival in high-grade osteosarcoma: improvement over 21 years at a single institution. Ann Oncol. 2010;21:1366–1373.
14. Ferrari S, Ruggieri P, Cefalo G, et al. Neoadjuvant chemotherapy with methotrexate, cisplatin, and doxorubicin with or without ifosfamide in nonmetastatic osteosarcoma of the extremity: an Italian sarcoma group trial ISG/OS-1. J Clin Oncol. 2012; 30: 2112–2118.
15. Anninga JK, Gelderblom H, Fiocco M, et al. Chemotherapeutic adjuvant treatment for osteosarcoma: where do we stand? Eur J Cancer 2011; 47: 2431-45.
16. Whelan JS, Bielack SS, Marina N, et al. EURAMOS-1, an international randomised study for osteosarcoma: results from pre-randomisation treatment. Ann Oncol. 2015; 26: 407-14.
17. JK, Gelderblom H, Fiocco M, et al. Chemotherapeutic adjuvant treatment for osteosarcoma: where do stand? Eur J Cancer. 2011; 47: 2431–2445.
18. S, Kempf-Bielack B, Von Kalle T, et al. Controversies in childhood osteosarcoma. Minerva Pediatr. 2013; 65: 125–148.
19. Luetke A, Meyers PA, Lewis I, et al. Osteosarcoma treatment–Where do we stand? A state of the art review.Cancer Treat Rev 2014; 40: 523–32.
20. Ferrari S, Serra M. An update on chemotherapy for osteosarcoma. Expert Opin Pharmacother. 2015; 16: 2727-36.
21. Su W, Lai Z, Wu F, et al. Clinical efficacy of preoperative chemotherapy with or without ifosfamide in patients with osteosarcoma of the extremity: metaanalysis of randomized controlled trials. Med Oncol 2015; 32: 481.
22. Meyers PA, Schwartz CL, Krailo M, et al. Osteosarcoma: randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate. J Clin Oncol. 2005; 23: 2004-11.
23. Meyers PA, Schwartz CL, Krailo, et al. Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival--a report from the Children's Oncology Group. J Clin Oncol. 2008; 26: 633-8.
24. Marina NM, Smeland S, Bielack SS, et al. Comparision of MAPIE versus MAP in patients with a poor response to preoperative chemotherapy for newly diagnosed high-grade osteosarcoma (EURAMOS-1): an open label, international, randomised controlled trial. Lancet Oncol. 2016; 17: 1396-1408.

25. Kager L, Pötschger U, Bielack S. Review of mifamurtide in the treatment of patients with osteosarcoma. Ther Clin Risk Manag. 2010; 6: 279-86.
26. Bielack SS, Smeland S, Whelan JS, et al. Methotrexate, Doxorubicin, and Cisplatin (MAP) Plus Maintenance Pegylated Interferon Alfa-2b Versus MAP Alone in Patients With Resectable High-Grade Osteosarcoma and Good Histologic Response to Preoperative MAP: First Results of the EURAMOS-1 Good Response Randomized Controlled Trial. J Clin Oncol. 2015; 33: 2279-87.
27. Ebb D, Meyers P, Grier H, et al. Phase II trial of trastuzumab in combination with cytotoxic chemotherapy for treatment of metastatic osteosarcoma with human epidermal growth factor receptor 2 overexpression: a report from the children's oncology group. J Clin Oncol 2012; 30: 2545-51.
28. Grignani G, Palmerini E, Dileo P, et al. A phase II trial of sorafenib in relapse and unresectable high-grade osteosarcoma after failure of standard multimodal therapy: an Italian Sarcoma Group Study. Ann Oncol 2012: 23: 508-16.
29. Grignani G, Palmerini E, Ferraresi V, et al. Sorafenib and everolimus for patients with unresectable high-grade osteosarcoma progressing after standard treatment: a non-randomised phase 2 clinical trial. Lancet Oncol 2015; 16: 98-107.
30. Piperno-Neumann S, Le Deley MC, Rédini F, et al. Zoledronate in combination with chemotherapy and surgery to treat osteosarcoma (OS2006): a randomised, multicentre, open-label, phase 3 trial. Lancet Oncol 2016: 17: 1070-80.
31. Koirala P, Roth ME, Gill J, et al. Immune infiltration and PD-L1 expression in the tumor microenvironment are prognostic factors in osteosarcoma. Sci Rep 2016; 6: 30093.
32. Roth M, Linkowski M, Tarim J, et al. Ganglioside GD2 as therapeutic target for antibody-mediated therapy in patietns with osteosarcoma. Cancer 2014; 120: 548-54.
33. Yu AL, Gilman AL, Ozkaynak MF, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. N Engl J Med 2010; 363:1324–34.
34. Anderson ME. Update on Survival on Osteosarcoma. Orthop Clin North Am. 2016; 47: 283-92.
35. Duchman KR, Gao Y, Miller BJ. Prognostic factors for survival in patients with high-grade osteosarcoma using the Surveillance, Epidemiology, and End Results (SEER) Program database. Cancer Epidemiol 2015; 39(4): 593–9.

36. Peris-Bonet R, Salmeron D, Martinez-Beneito MA, Galceran J, Marcos-Gragera R, Felipe S, et al. Childhood cancer incidence and survival in Spain. Ann Oncol 2010; 21: 103-110
37. Peris Bonet R, Fuentes García S. Cáncer infantil en España. Estadísticas 1980-2012. Registro Nacional de Tumores Infantiles (RNTI-SEHOP). Valencia: Universitat de Valencia: 2013. http://www.uv.es/rnti. Fecha acceso 11 Enero 2017
38. Tai E, Buchanan N, Westervelt L, et al. Treatment setting, clinical trial enrollment, and subsequent outcomes among adolescents with cancer: a literature review. Pediatrics 2014; 133: S91-7.
39. Muñoz A, Alfaro J, Pardo N, et al. Long-term results of the Spanish Protocol SO-95 for the treatment of non-metastatic high-grade osteosarcoma of the extremities in children. Clin Transl Oncol. 2009; 11: 387-92.
40. Bishop MW, Janeway KA, Gorlick R. Future directions in the treatment of osteosarcoma. Curr Opin Pediatr. 2016; 28: 26-33.
41. de Bono JS, Ashworth A. Translating cancer research into targeted therapeutics. Nature. 2010; 467: 543-9.
42. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144: 646-74.
43. Jokinen E, Koivunen JP. MEK and PI3K inhibition in solid tumors: rationale and evidence to date. Ther Adv Med Oncol 2015; 7: 170-80.
44. Huang WC, Hung MC. Induction of Akt activity by chemotherapy confers acquired resistance. J Formos Med Assoc. 2009; 108: 180-94.
45. Hartmann W, Kuchler J, Koch A, Friedrichs N, Waha A, Endl E, et al. Activation of phosphatidylinositol-3'-kinase/AKT signaling is essential in hepatoblastoma survival. Clin Cancer Res. 2009; 15: 4538-45.
46. Opel D, Poremba C, Simon T, Debatin KM, Fulda S. Activation of Akt predicts poor outcome in neuroblastoma. Cancer Res. 2007; 67: 735-45.
47. Lim HJ, Crowe P, Yang JL. Current clinical regulation of PI3K/PTEN/ Akt/mTOR signalling in treatment of human cancer. J Cancer Res Clin Oncol. 2015; 141: 671-89.
48. Loh AH, Brennan RC, Lang WH, Hickey RJ, Malkas LH, Sandoval JA. Dissecting the PI3K Signaling Axis in Pediatric Solid Tumors: Novel Targets for Clinical Integration. Front Oncol. 2013; 3: 93.
49. Santarpia L, Lippman SM, El-Naggar AK. Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. Expert Opin Ther Targets. 2012; 16: 103-19.

50. MacDonald B, Tamai K, He X. Wnt/ β-catenin signaling: components, mechanisms and diseases. Dev Cell. 2009; 17:9-26.
51. Freeman SS, Allen SW, Ganti R, Wu J, Ma J, Su X, et al. Copy number gains in EGFR and copy number losses in PTEN are common events in osteosarcoma tumors. Cancer. 2008; 113: 1453-61.
52. Choy E, Hornicek F, MacConaill L, Harmon D, Tariq Z, Garraway L, et al. High-throughput genotyping in osteosarcoma identifies multiple mutations in phosphoinositide-3-kinase and other oncogenes. Cancer. 2012; 118: 2905-14.
53. Chandhanayingyong C, Kim Y, Staples JR, Hahn C, Lee FY. MAPK/ERK Signaling in Osteosarcomas, Ewing Sarcomas and Chondrosarcomas: Therapeutic Implications and Future Directions. Sarcoma. 2012;2012:404810.
54. Tian J, He H, Lei G. Wnt/β-catenin pathway in bone cancers. Tumour Biol. 2014; 35: 9439-45.
55. Chang L, Shrestha S, LaChaud G, e al. Review of microRNA in osteosarcoma and chrondrosarcoma. Med Oncol. 2015; 32: 172.