Molecular Biology of Pediatric Bone Sarcomas 2

Ana Patiño-Garcia, Marta Zalacain-Diez, and Fernando Lecanda

Abstract Genetic studies can help in diagnosis, prognosis and treatment of pediatric bone sarcoma patients. On the basis of recent discoveries, new drugs (targeted therapies) to help cure these patients are being developed.

Osteosarcoma

Genetic Alterations

The molecular pathways involved in osteosarcoma development are complex and have not been fully explored, and their implication in the development and prognosis of this childhood tumour are not well understood. Even though certain clinical markers are clearly associated with prognosis, their value is limited by the fact that they become evident in stages of the tumoural process which are advanced: development of metastases, relapse and response to neo-adjuvant chemotherapy. It is becoming imperative to determine early molecular markers that allow for a more rationale use of chemotherapy, for the development of new effective treatments and for the stratification of patients according to risk.[1]

In this chapter, we will give a concise description of the pathways most frequently associated with osteosarcoma development and pathways that have been proved to have a prognostic value.

Angiogenesis

The vascular endothelial growth factor (VEGF) stimulates microvascular growth and has an outstanding role in the development of certain tumours (breast, colon, etc.) by increasing the supply of nutrients and blood to them. The use of anti-angiogenic agents

Ana Patiño-Garcia (✉)
Laboratory and Department of Pediatrics, University Clinic of Navarra, Irunlarrea SN, Pamplona, Navarra 31080, Spain
e-mail: apatigar@unav.es

J. Cañadell and M. San-Julian (eds.), *Pediatric Bone Sarcomas: Epiphysiolysis Before Excision*,
DOI: 10.1007/978-1-84882-130-9_2,

(inhibitors of the VEGF pathway) is controversial in the case of osteosarcoma, and reports show contradictory results.[2,3] Therefore, the utility of this and other related molecules [pigment epithelium derived factor (PEDF)] as therapeutic targets in osteosarcoma and their synergic effect with chemotherapy has yet to be determined, with studies already in progress.[4]

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are enzymes that are physiologically involved in tissue remodelling and angiogenesis. Excessive production of MMPs, whether as a result of increased transcription of coding genes or of a lack of inhibitors, is important in the process of invasion and metastasis. MMP9, a member of this family, seems to have a prominent role in bone remodelling diseases like osteosarcoma, and several publications have shown that MMP9 overexpression is a poor prognostic factor in osteosarcoma (with an increase in metastatic potential and reduced 5-year overall survival).[5,6] MMP9 can be repressed by a variety of molecules, a fact that makes it an interesting target for attempts to decrease the invasive potential of tumour cells. Indeed, this effect has been demonstrated in animal models and cultured cells.

P Glycoprotein

P glycoprotein (P-gp), which is codified by the multidrug resistance 1 (MDR1) gene, is a membrane molecule involved in drug transport. For more than a decade P-gp overexpression has been known to be a poor prognostic factor in osteosarcoma.[7] There are various reasons for this, one of them being that it is the mechanism by which osteosarcoma cells become resistant to doxorubicin, a prime cytostatic drug in the standard chemotherapy for this pediatric tumour.[8]

Some authors have identified a link between P-gp and p53 overexpression. The p53 molecular pathway is another pathway frequently altered in this type of tumour. According to this model, those patients whose tumours have an altered coexpression of P-gp and p53 would have significantly reduced survival and a more unfavourable Enneking stage.[9]

Cell Cycle Control

Alteration of the different components of the pathway of cell cycle control, particularly those of the p53 and retinoblastoma (RB1) pathways, seems to be the hallmark of the carcinogenic process underlying pediatric osteosarcoma: an alteration in the p53 pathway, the RB1 pathway or both has been detected in most tumours.[10]

The p53 Pathway

Alterations that lead to inactivation of the p53 tumour suppression gene are frequently found in sporadic human tumours. The result is a loss of control of the cell cycle and the DNA repair mechanisms (Fig. 2.1). Although there is considerable published evidence suggesting that the p53 protein has a role in the development of both sporadic osteosarcomas and those associated with the Li–Fraumeni syndrome,[11] the prognostic value of such alterations has not been definitely established.[12,13]

The RB1 Pathway

The cell cycle control pathway that includes the retinoblastoma gene, RB1, is frequently altered in human tumours, especially in osteosarcomas[14] (Fig. 2.1). As described later, the loss of genetic material affecting the long arm of chromosome 13 (13q14) is a frequent genetic event in primary osteosarcomas, and this loss indicates the presence of a tumour suppressor gene at this chromosomal location. This suppressor has turned out to be RB1.[15] About 50–70% of osteosarcomas have a hemizygous deletion affecting the RB1 gene,[16,17]

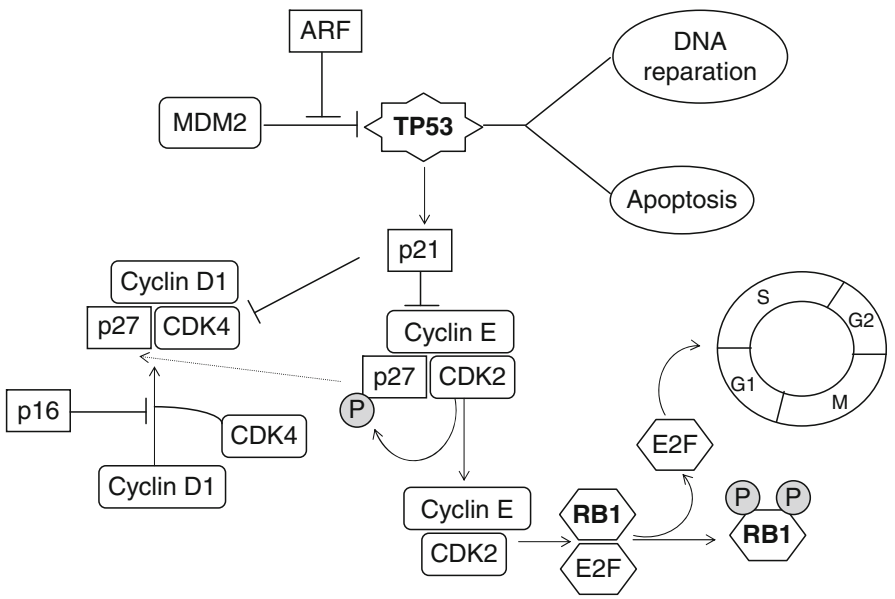

Fig. 2.1 Schematic representation of the cell cycle control mediated by TP53 and RB1

structural rearrangements (30%)[18] or point mutations of the gene (10%).[19] The presence of alterations at the RB1 locus can be considered an early marker of malignancy and of unfavourable prognosis and, in addition, RB1 alterations are more frequently encountered in high-grade than in low-grade osteosarcomas.[20,21] However, as with many of the molecular markers of this specific tumour, this prognostic association is not always found.[22]

Growth Factors

1. *Members of the WNT Family (Wingless-Type)*. The WNT signalling pathway controls the normal bone formation during embryogenesis and bone homeostasis in the adult. The pathway has been investigated in the context of the osteosarcoma model in different publications. Recent data suggest that the WNT pathway may have a paracrine and autocrine effect involved in the metastatic potential of osteosarcoma,[23,24] although these data remain to be reproduced.
2. *Her-2/neu (Epidermal Growth Factor Receptor)*. The overexpression of this tyrosine kinase is considered a poor prognostic factor in various types of carcinoma, since it is related with the tumour growth and the metastatic process. Even though the involvement of Her-2 in osteosarcoma has not been unequivocally established, and different reports show controversial results,[25,26] it is an attractive therapeutic target since an antagonist, Trastuzumab/Herceptin, is already available in the clinical setting.

There are many other genes of interest and are under investigation. One of these is *ezrin*, because the encoded protein is involved in cell-to-cell interaction and in signal transduction. Overexpression of ezrin is a promoter factor for metastases, probably through the mitogen-activated protein kinases (MAPK) signalling pathway.[27,28]

The fact that the peak incidence of osteosarcoma ovelaps with the pubertal growth spurt may indicate that insulin-like growth factor-I (*IGF-I*) and its receptor play a role in the pathogenesis of this disease. The IGF-I growth factor acts as a mitogen on both murine and human osteosarcoma cells, and osteosarcoma cell lines are dependent on IGF-I for in vitro growth. Even though the levels of IGF-I and its binding protein (IGFBP3) are not increased in osteosarcoma patients, other members of the IGF-I axis could be involved in the development and progression of osteosarcoma.[29,30]

Chromosomal Alterations

Conventional karyotype analyses show that, as a general rule, osteosarcomas have complex altered karyotypes with multiple structural and numerical aberrations. The most frequently encountered alterations in primary tumours (as opposed to cell lines) are the duplication of chromosome 1, loss of chromosomes 9, 10, 13 (RB1 locus) and 17 (TP53 locus), and either partial or complete loss of chromosome 6. The common findings of most

cytogenetic studies indicate the presence of frequent breaks and aberrations at the following locations: 1p11-13, 1q11-12, 1q21-22, 11p15, 12p13, 17p11-13, 19q13 and 22q11-13.[31] Studies based on metaphase comparative genomic hybridization (CGH) have been a useful tool to unveil and characterise such complex karyotypes with high resolution and have identified high copy number regions or amplifications at 8q12-q21.3, 8q22-q23 (MYC gene) and at 17p11.2-17p12.[32] The chromosomal regions that are more frequently gained or lost have been carefully identified and reviewed.[33–35] Work by Ozaki and colleagues establishes that the gain or loss of some of these regions, either as isolated aberrations or as specific combinations, might have prognostic value.[36]

Ewing's Sarcoma Tumours

Introduction

Tumours within the Ewing's sarcoma family (EFTs) constitute the second most frequent type of bone-/soft-tissue sarcoma in children and adolescents.[37,38] The family comprises classical Ewing's sarcoma, peripheral primitive neuroectodermal tumours, and Askin's tumour, all of which are highly aggressive and frequently metastatic.[39] Tumours often appear in tubular bones of the appendicular skeleton (58%), although they also arise in the axial skeleton (33%) and at extraosseous sites.[40] Histologically, Ewing's tumours are characterised by the presence of small round cells with prominent and regular nuclei containing inconspicuous nucleoli, indistinct cytoplasm[38] and various degrees of neural and endothelial differentiation.[41]

Until the advent of differential molecular techniques, an unambiguous diagnosis required experienced pathological assessment. None of the markers used in conventional immunohistochemistry showed complete specificity. The transmembrane glycoprotein MIC2/CD99, the most specific marker so far, is expressed in more than 98% of EFTs.[42] Other tumours, including rabdomyosarcoma and lymphoblastic lymphoma, also present positive immunostaining. Depending on the degree of neuroectodermal differentiation, EFTs may also express neural markers, including S-100 synaptophysin, neural-specific enolase, CD57 and various neurofilaments.[43] The best tools for an unambiguous diagnosis are fluorescence in situ hybridisation and RT-PCR using a combination of primers targeting the underlying chromosomal translocations.[44]

Most of the progress over the last few decades has improved pathological definition and staging.[45] Despite a parallel improvement in treatment by multi-modal combination of surgery with chemo- and radio-therapy, the 5-year survival rate remains close to 50% for patients with primary tumours,[46] only 25% for patients with lung metastasis, and the prognosis for patients with bone or bone marrow metastasis is even worse.[47] The tumour also exhibits a strong tendency to metastasise through hematogenous spread to the lungs and frequently to the skeleton. Thus, in addition to the stage, location and size of the tumour, metastasis is a reliable prognosis factor indicative of poor prognosis.

2

Molecular Biology

The demonstration that fusion proteins are the "culprits" of the transforming events giving rise to hematological malignancies provided a strong rationale for investigation of fusion proteins in other tumours.

EFTs belong to a growing family of sarcomas characterised by specific reciprocal chromosomal translocations, which generate fusion genes: the neoplasm has a relatively simple cytogenetic background.[48]

At present, more than 15 different fusion proteins have been identified in EFTs. Chromosomal translocation t(11;22) q(24;q12) produces gene fusions between the amino terminus domain of EWS and the C-terminal region of a member belonging to the ETS family of transcription factors. Fusions resulting from the translocation give rise to functionally aberrant transcription factors potentially able to drive transformation in a permissive cellular context.

In 85% of Ewing's tumours, Friend Leukemia Integration 1 transcription factor (FLI-1) is the EWS partner which accounts for the different fusion subtypes observed.[49] Depending on the juxtaposed exons assembled by EWS and FLI-1 breakpoints, several subtypes have been described. Most tumours contain EWS/FLI-1 fusion types 1 (60%) and 2 (25%) which have been associated with different clinical features and prognosis.[50,51]

The FLI-1 gene displays a restricted pattern of expression, mainly in hematopoietic cells, and at lower levels in heart lung and ovaries.[52] During development FLI-1 is also expressed in neural crest-derived mesenchymal lineage and endothelial cells, which is consistent with its role in vasculogenesis and hematopoiesis.[53]

In contrast to FLI-1, the EWS gene encodes a ubiquitously expressed protein which belongs to the TET family of RNA binding proteins.[49,54] The TET family also includes TAFII68 (or TAF15)[55,56] and FUS (or TLS),[57] both of which share similar structural domains with EWS and have been found to form gene fusions with non-ETS transcription factors, giving rise to non-Ewing sarcomas such as myxoid liposarcomas,[58] myxoid chondrosarcomas[59] and desmoplastic small round-cell tumours,[60] with different histopathological features.

EWS contains three arginine–glycine–glycine (RGG) rich motifs that participate in RNA biogenesis and processing through interaction with proteins of the basal transcription machinery, including TFIID, RNA polymerase II[61,62] and coactivators such as CBP/p300.[55,54] In addition, TET proteins interact with splicing proteins. Indeed, EWS/FLI-1 has been shown to bind the splicing factor U1C and to modulate splicing activity.[62–64] Interaction with other proteins has also been described, for example, EWS interacts with BARD1, although the relevance of this to tumourogenesis has not been elucidated.[65]

Most studies have focused on the function of EWS as a transcription factor. The amino terminal domain of EWS contains a glutamine-rich N-terminal region containing a potent transcriptional activation domain[66,67] that, when fused to the DNA-binding domain of FLI-1, generates an aberrantly active transcription factor capable of specifically binding DNA.[68] Since the majority of fusion subtypes do not encompass the RGG domain, most studies have not assessed the effects of EWS–ETS fusions in RNA biogenesis.

All members of the ETS family of transcription factors are characterised by a common DNA-binding domain. Fusions of EWS with other ETS members have been described,[69]

including ERG (in 10% of Ewing's tumours)[70] and with other fairly rare partners such as ETV1,[71] ETV4[72] and FEV.[73] The fact that different combinations of EWS/ETS give rise to Ewing's tumours of similar histopathology suggests that the potent transactivation domain of EWS, through interaction with other unknown proteins, is critical for transformation. Complementary to this view, the DNA binding domains of ETS proteins are highly homologous and all recognise targets containing a similar core sequence, and therefore, despite their differences, all EWS–ETS fusion proteins can be expected to act in a similar manner, disturbing a closely regulated pattern of gene transcription, with the consequent formation of Ewing's tumours.

The Oncoprotein EWS/FLI-1 as a Paradigm

The discovery of EWS/FLI-1 underscores the attractiveness of an approach to research looking to reveal common mechanisms in many tumours arising from specific translocations. Depending on the cellular background, EWS/FLI-1 induces a variety of responses that include transformation, senescence, differentiation, and cell lineage commitment.

The presence of neural markers and the diverse sites of origin have frequently led to the assumption or hypothesis that EFTs evolve from a cell type with multilineage differentiation potential.

In vitro experiments revealed that the chimeric EWS/FLI-1 acts as a potent repressor of normal cell fate. In murine primary marrow-derived stromal cells, EWS/FLI-1 represses osteogenic and adipogenic programs.[74,75] Similarly, myogenic differentiation was suppressed by the chimeric protein in a murine multipotent mesenchymal cell line.[74] In contrast, in other cellular backgrounds, EWS/FLI-1 dictates cell lineage commitment by redirecting cell lineage towards a neural-like phenotype.[76] Differentiation towards the neuroectodermal phenotype typical of Ewing's tumours, with the acquisition of a small round cell morphology, has been obtained in a fibroblastic cell line.[77] Similarly, in neuroblastoma, Hela, and rhabdomyosarcoma cell lines, the forced expression of EWS-FLI1 resulted in the acquisition of neural phenotypic traits.[78] Consistent with the role of FLI-1, in a separate rhabdomyosarcoma cell line, the expression of neural crest phenotypic markers was induced by EWS/FLI-1.[79]

In addition to repression of normal cell fate, EWS/FLI-1 is thought to induce cell-specific oncogenesis in a transformation-permissive cellular background. However, many cell lines, including Rat-1 fibroblasts, Ncm1, CTR, and the NIH 3T3-derived cell line YAL-7, have been found to be refractory to transformation.[80] Single-step oncogenesis has been reported in murine primary cells.[66,77,81,82] This finding can be attributed to a better transformation potential of rodent cells compared to human cells.[83,84] In the study by Castillero-Trejo et al, tumourogenicity increased with cell passage in culture and other secondary events, including p53 deletion.[81] These murine bone-derived cells expressing EWS/FLI-1 showed formation of sarcomatous tumours in syngeneic mice. Riggi et al reported that a single event of transduction with EWS/FLI-1 was sufficient to reconstitute the hallmarks of Ewing sarcomagenesis in a murine model.[82] In contrast, Riggi et al were unable to reproduce the same findings in primary human cells.[85] Studies of a human model have recently

provided strong experimental evidence suggestive that the originating cell type is of mesenchymal lineage.[86]

In the mouse, background tumour suppressor pathways, including p16/p19 and p53, may be overcome by unknown factors. In human cells, however, additional mutations may be required to circumvent the strong tumour suppressor program. Indeed, other cytogenetic events[87] and additional mutations have been found in 20–30%[88–90] of human Ewing's tumours. An alternative suggestion recently put forward is that deregulated progenitor cells present in adult tissues are the cancer-initiating cells that are able to sustain tumour growth in vivo.[91,92] It is possible that the target for the transformation event driven by EWS/FLI-1 is an unidentified progenitor cell yet to be determined.

Target Genes

One of the main goals in the study of Ewing sarcomagenesis has been to identify downstream target genes regulated by EWS/FLI-1. Global transcriptomic analysis in combination with other techniques has elucidated several potential target genes involved in the genetic program driven by the fusion protein. The genes found are involved in neural differentiation, cell proliferation and anti-apoptotic functions. However, because the studies differ significantly depending on the chosen cellular model, it has been difficult to discern which EWS/FLI-1-responsive genes are associated with the initiation and maintenance of tumours.

The most compelling identification of a critical target of EWS/FLI-1 is that of the homeodomain protein NKX2.2.[93] NKX2.2 is transcriptional repressor involved in neural-cell differentiation. Induction of NKX2.2 is necessary for oncogenic transformation and represents a potential Ewing diagnostic marker. The protein's strong repressive function is mediated by a HDAC-dependent mechanism.[93,94]

Id2, a helix–loop–helix transcription factor without the DNA-binding domain, has been found to be upregulated by EWS/FLI-1 in Ewing's tumours. Through interaction with a variety of cell cycle proteins including p21 and Rb tumour suppressors,[95,96] Id2 is able to promote cell proliferation.

Other potential target genes transcriptionally upregulated by EWS/FLI-1 include PDGF-C,[97] which is expressed in more than 60% of tumours, CCND1[98,99] and c-Myc.[100,101]

hTERT, the catalytic subunit of telomerase and one of the hallmarks of many tumours, has also been found to be upregulated in approximately 80% of Ewing's samples. The upregulation is an indirect effect of EWS/FLI-1[102] through the recruitment of an unknown ancillary protein. Similarly, key tumour suppressors such as p57,[100] p21[103] and TGFBRII[104,105] were found to be downregulated in Ewing's tumours.

Interestingly, IGFBP-3 is a direct target of the fusion protein: EWS/FLI-1 binds to the IGFBP-3 promoter both in vitro and in vivo,[106] and the consequent repression leads to increased Akt activity and decreased apoptotic activity. Similarly, the IGF-1/IGF-1R axis, which is frequently required for Ewing's tumour cell growth, promotes cell survival through the Akt pathway.[107,108] Different pharmacological strategies targeting IGF1R are currently being explored for the treatment of Ewing's tumours.[109,110]

Recently, the combination of the techniques of transcriptomic analysis with high throughput chromatin immunoprecipitation analysis has validated previous research on EWS/FLI-1 by identifying previously reported genes (NKX2.2, ID2 and CCND1) and identifiying additional biologically relevant targets, including NROB1[111–113] and GAS1. The role of these targets in Ewing sarcomagenesis is yet to be determined.

Future Directions

The biology of EFTs remains an attractive experimental platform to understand critical questions regarding the development of both EFTs and a variety of other sarcomas. Despite the remarkable progress over the last two decades, there are still several questions that have not been rigorously addressed. Amongst these are the clear definition of the permissive cell and the specific time and microenvironment required for transformation. Similarly, the critical molecular events driven by the chimeric EWS/FLI-1 protein to initiate and maintain Ewing's tumours remain to be systematically dissected in an appropriate model. To this end, an animal model faithfully reproducing the spatio-temporal development of Ewing's sarcomas would be an invaluable tool. Only with precise knowledge at the cellular and molecular levels can we expect to elucidate critical target genes, and thereby facilitate the development of more focused and efficient therapies.

Referenes

1. Clark JC, Dass CR, Choong PF. A review of clinical and molecular prognostic factors in osteosarcoma. *J Cancer Res Clin Oncol*. 2008;134:281–297.
2. Ek ET, Ojaimi J, Kitagawa Y, Choong PF. Does the degree of intratumoral microvessel density and VEGF expression have prognostic significance in osteosarcoma. *Oncol Rep*. 2006;16:17–23.
3. Kreuter M, Bieker R, Bielack SS, et al. Prognostic relevance of increased angiogenesis in osteosarcoma. *Clin Cancer Res*. 2004;10:8531–8537.
4. Stempak D, Gammon J, Halton J, Moghrabi A, Koren G, Baruchel S. A pilot pharmacokinetic and antiangiogenic biomarker study of celecoxib and low-dose metronomic vinblastine or cyclophosphamide in pediatric recurrent solid tumors. *J Pediatr Hematol Oncol*. 2006;28:720–728.
5. Foukas AF, Deshmukh NS, Grimer RJ, Mangham DC, Mangos EG, Taylor S. Stage-IIB osteosarcomas around the knee A study of MMP-9 in surviving tumor cells. *J Bone Joint Surg Br*. 2002;84:706–711.
6. Kido A, Tsutsumi M, Iki K, et al. Overexpression of matrix metalloproteinase (MMP)-9 correlates with metastatic potency of spontaneous and 4-hydroxyaminoquinoline 1-oxide (4-HAQO)-induced transplantable osteosarcomas in rats. *Cancer Lett*. 1999;137:209–216.
7. Pakos EE, Ioannidis JP. The association of P-glycoprotein with response to chemotherapy and clinical outcome in patients with osteosarcoma A meta-analysis. *Cancer*. 2003 August 1;98(3): 581–589.
8. Baldini N, Scotlandi K, Serra M, et al. P-glycoprotein expression in osteosarcoma: a basis for risk-adapted adjuvant chemotherapy. *J Orthop Res*. 1999;17:629–632.
9. Park YB, Kim HS, Oh JH, Lee SH. The co-expression of p53 protein and P-glycoprotein is correlated to a poor prognosis in osteosarcoma. *Int Orthop*. 2001;24:307–310.
10. Kansara M, Thomas DM. Molecular pathogenesis of osteosarcoma. *DNA Cell Biol*. 2007;26:1–18.

11. Soussi T, Leblanc T, Baruchel A, Schaison G. Germline mutations of the p53 tumor-suppressor gene in cancer-prone families: a review. *Nouv Rev Fr Hematol*. 1993;35:33–36.
12. Kaseta MK, Khaldi L, Gomatos IP, et al. Prognostic value of bax, bcl-2, and p53 staining in primary osteosarcoma. *J Surg Oncol*. 2008;97:259–266.
13. Wunder JS, Gokgoz N, Parkes R, et al. TP53 mutations and outcome in osteosarcoma: a prospective, multicenter study. *J Clin Oncol*. 2005;23:1483–1490.
14. Belchis DA, Gocke CD, Geradts J. Alterations in the rb, p16, and cyclin d1 cell cycle control pathway in osteosarcomas. *Pediat Pathol Mol Med*. 2000;19:377–389.
15. Yamaguchi T, Toguchida J, Yamamuro T, et al. Allelotype analysis in osteosarcomas: frequent allele loss on 3q, 13q, 17p, and 18q. *Cancer Res*. 1992;52:2419–2423.
16. Wadayama B, Feugeas O, Guriec N, et al. Loss of heterozygosity of the RB gene is a poor prognostic factor in patients with osteosarcoma. *J Clin Oncol*. 1996;14:467–472.
17. Benassi MS, Molendini L, Gamberi G, et al. Alteration of pRb/p16/cdk4 regulation in human osteosarcoma. *Int J Cancer*. 1999;84:489–493.
18. Miller CW, Aslo A, Won A, Tan M, Lampkin B, Koeffler HP. Alterations of the p53, Rb and MDM2 genes in osteosarcoma. *J Cancer Res Clin Oncol*. 1996;122:559–565.
19. Wadayama B, Toguchida J, Shimizu T, et al. Mutation spectrum of the retinoblastoma gene in osteosarcomas. *Cancer Res*. 1994;54:3042–3048.
20. Patiño-García A, Piñeiro ES, Díez MZ, Iturriagagoitia LG, Klüssmann FA, Ariznabarreta LS. Genetic and epigenetic alterations of the cell cycle regulators and tumor suppressor genes in pediatric osteosarcomas. *J Pediatr Hematol Oncol*. 2003;25:362–367.
21. Wunder JS, Czitrom AA, Kandel R, Andrulis IL. Analysis of alterations in the retinoblastoma gene and tumor grade in bone and soft-tissue sarcomas. *J Natl Cancer Inst*. 1991;83:194–200.
22. Heinsohn S, Evermann U, Zur Stadt U, Bielack S, Kabisch H. Determination of the prognostic value of loss of heterozygosity at the retinoblastoma gene in osteosarcoma. *Int J Oncol*. 2007;30:1205–1214.
23. Chen K, Fallen S, Abaan HO, Hayran M, et al. WNT10b induces chemotaxis of osteosarcoma and correlates with reduced survival. *Pediatr Blood Cancer*. 2008;51:349–355.
24. Hoang BH, Kubo T, Healey JH, et al. Expression of LDL receptor-related protein 5 (LRP5) as a novel marker for disease progression in high-grade osteosarcoma. *Int J Cancer*. 2004;109:106–111.
25. Rakesh Kumar V, Gupta N, Kakkar N, Sharma SC. Prognostic and predictive value of c-erbB2 overexpression in osteogenic sarcoma. *J Cancer Res Ther*. 2006;2:20–23.
26. Zhou H, Randall RL, Brothman AR, Maxwell T, Coffin CM, Goldsby RE. Her-2/neu expression in osteosarcoma increases risk of lung metastasis and can be associated with gene amplification. *J Pediatr Hematol Oncol*. 2003;25:27–32.
27. Ferrari S, Zanella L, Alberghini M, Palmerini E, Staals E, Bacchini P. Prognostic significance of immunohistochemical expression of ezrin in non-metastatic high-grade osteosarcoma. *Pediatr Blood Cancer*. 2008;50:752–756.
28. Park HR, Jung WW, Bacchini P, Bertoni F, Kim YW, Park YK. Ezrin in osteosarcoma: comparison between conventional high-grade and central low-grade osteosarcoma. *Pathol Res Pract*. 2006;202:509–515.
29. Chavez Kappel C, Velez-Yanguas C, Hirschfeld S, Helman LJ. Human osteosarcoma cell lines are dependent on insulin-like growth factor for in vitro growth. *Cancer Res*. 1994;54:2803–2807.
30. Rodriguez-Galindo C, Poquette CA, Daw NC, Tan M, Meyer WH, and Cleveland JL. Circulating concentrations of IGF-I and IGFBP-3 are not predictive of incidence or clinical behavior of pediatric osteosarcoma. *Med Pediatr Oncol*. 2001;36:605–611.
31. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. *Cancer Genet Cytogenet*. 2003;145:1–30.

32. Squire JA, Pei J, Marrano P, et al. High-resolution mapping of amplifications and deletions in pediatric osteosarcoma by use of CGH analysis of cDNA microarrays. *Genes Chromosomes Cancer*. 2003;38:215–225.
33. Forus A, Weghuis DO, Smeets D, Fodstad O, Myklebost O, Geurts van Kessel A. Comparative genomic hybridization analysis of human sarcomas: II. Identification of novel amplicons at 6p and 17p in osteosarcomas. *Genes Chromosomes Cancer*. 1995;14:15–21.
34. Tarkkanen M, Karhu R, Kallioniemi A, et al. Gains and losses of DNA sequences in osteosarcomas by comparative genomic hybridization. *Cancer Res*. 1995;55:1334–1338.
35. Zielenska M, Marrano P, Thorner P, et al. High-resolution cDNA microarray CGH mapping of genomic imbalances in osteosarcoma using formalin-fixed paraffin-embedded tissue. *Cytogenet Genome Res*. 2004;107:77–82.
36. Ozaki T, Schaefer KL, Wai D, et al. Genetic imbalances revealed by comparative genomic hybridization in osteosarcomas. *Int J Cancer*. 2002;102:355–365.
37. Gurney JG, Davis S, Severson RK, Fang JY, Ross JA, Robison LL. Trends in cancer incidence among children in the U.S. *Cancer*. 1996;78:532–541.
38. Arndt CA, Crist WM. Common musculoskeletal tumors of childhood and adolescence. *N Engl J Med*. 1999;341:342–352.
39. de Alava E, Gerald WL. Molecular biology of the Ewing's sarcoma/primitive neuroectodermal tumor family. *J Clin Oncol*. 2000;18:204–213.
40. Grier HE. The Ewing family of tumors Ewing's sarcoma and primitive neuroectodermal tumors. *Pediatr Clin North Am*. 1997;44:991–1004.
41. Franchi A, Pasquinelli G, Cenacchi G, et al. Immunohistochemical and ultrastructural investigation of neural differentiation in Ewing sarcoma/PNET of bone and soft tissues. *Ultrastruct Pathol*. 2001;25:219–225.
42. Kovar H, Dworzak M, Strehl S, et al. Overexpression of the pseudoautosomal gene MIC2 in Ewing's sarcoma and peripheral primitive neuroectodermal tumor. *Oncogene*. 1990;5:1067–1070.
43. Ushigome SMR, Sorensen PH. Ewing sarcoma/Primitive neuroectodermal tumor (PNET). In: Christopher DM, Fletcher KKU, Fredrik M, eds. *Pathology and Genetics of Tumors of Soft Tissue and BoneWorld Health Organization Classification of Tumors.* Lyon: Pathology and Genetics of Tumors of Soft Tissue and Bone International Agency for Research on Cancer; 2002.
44. Peter M, Gilbert E, Delattre O. A multiplex real-time PCR assay for the detection of gene fusions observed in solid tumors. *Lab Invest*. 2001;81:905–912.
45. Burchill SA. Ewing's sarcoma: diagnostic, prognostic, and therapeutic implications of molecular abnormalities. *J Clin Pathol*. 2003;56:96–102.
46. Paulussen M, Ahrens S, Craft AW, et al. Ewing's tumors with primary lung metastases: survival analysis of 114 (European Intergroup) Cooperative Ewing's Sarcoma Studies patients. *J Clin Oncol*. 1998;16:3044–3052.
47. Cotterill SJ, Ahrens S, Paulussen M, et al. Prognostic factors in Ewing's tumor of bone: analysis of 975 patients from the European Intergroup Cooperative Ewing's Sarcoma Study Group. *J Clin Oncol*. 2000;18:3108–114.
48. Mackall CL, Meltzer PS, Helman LJ. Focus on sarcomas. *Cancer Cell*. 2002;2:175–178.
49. Delattre O, Zucman J, Plougastel B, et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumors. *Nature*. 1992;359:162–165.
50. de Alava E, Kawai A, Healey JH, et al. EWS-FLI1 fusion transcript structure is an independent determinant of prognosis in Ewing's sarcoma. *J Clin Oncol*. 1998;16:1248–1255.
51. Zoubek A, Dockhorn-Dworniczak B, Delattre O, et al. Does expression of different EWS chimeric transcripts define clinically distinct risk groups of Ewing tumor patients. *J. Clin Oncol*. 1996;14:1245–1251.
52. Ben-David Y, Giddens EB, Letwin K, Bernstein A. Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. *Genes Dev*. 1991;5:908–918.

53. Melet F, Motro B, Rossi DJ, Zhang L, Bernstein A. Generation of a novel Fli-1 protein by gene targeting leads to a defect in thymus development and a delay in Friend virus-induced erythroleukemia. *Mol Cell Biol*. 1996;16:2708–2718.
54. Ohno T, Ouchida M, Lee L, Gatalica Z, Rao VN, Reddy ES. The EWS gene, involved in Ewing family of tumors, malignant melanoma of soft parts and desmoplastic small round cell tumors, codes for an RNA binding protein with novel regulatory domains. *Oncogene*. 1994;9: 3087–3097.
55. Bertolotti A, Lutz Y, Heard DJ, Chambon P, Tora L. hTAF(II)68, a novel RNA/ssDNA-binding protein with homology to the pro-oncoproteins TLS/FUS and EWS is associated with both TFIID and RNA polymerase II. *EMBO J*. 1996;15:5022–5031.
56. Aman P, Panagopoulos I, Lassen C, et al. Expression patterns of the human sarcoma-associated genes FUS and EWS and the genomic structure of FUS. *Genomics*. 1996;37:1–8.
57. Shing DC, McMullan DJ, Roberts P, et al. FUS/ERG gene fusions in Ewing's tumors. *Cancer Res*. 2003;63:4568–4576.
58. Crozat A, Aman P, Mandahl N, Ron D. Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. *Nature*. 1993;363:640–644.
59. Labelle Y, Zucman J, Stenman G, et al. Oncogenic conversion of a novel orphan nuclear receptor by chromosome translocation. *Hum Mol Genet*. 1995;4:2219–2226.
60. Ladanyi M, Gerald W. Fusion of the EWS and WT1 genes in the desmoplastic small round cell tumor. *Cancer Res*. 1994;54:2837–2840.
61. Petermann R, Mossier BM, Aryee DN, Khazak V, Golemis EA, Kovar H. Oncogenic EWS-Fli1 interacts with hsRPB7, a subunit of human RNA polymerase II. *Oncogene*. 1998;17:603–610.
62. Yang L, Chansky HA, Hickstein DD. EWS.Fli-1 fusion protein interacts with hyperphosphorylated RNA polymerase II and interferes with serine-arginine protein-mediated RNA splicing. *J Biol Chem*. 2000;275:37612–37618.
63. Knoop LL, Baker SJ. The splicing factor U1C represses EWS/FLI-mediated transactivation. *J Biol Chem*. 2000;275:24865–24871.
64. Knoop LL, Baker SJ. EWS/FLI alters 5′-splice site selection. *J Biol Chem*. 2001; 276:22317–22322.
65. Spahn L, Petermann R, Siligan C, Schmid JA, Aryee DN, Kovar H. Interaction of the EWS NH2 terminus with BARD1 links the Ewing's sarcoma gene to a common tumor suppressor pathway. *Cancer Res*. 2002;62:4583–4587.
66. May WA, Gishizky ML, Lessnick SL, et al. Ewing sarcoma 11;22 translocation produces a chimeric transcription factor that requires the DNA-binding domain encoded by FLI1 for transformation. *Proc Natl Acad Sci U S A*. 1993;90:5752–5756.
67. Lessnick SL, Braun BS, Denny CT, May WA. Multiple domains mediate transformation by the Ewing's sarcoma EWS/FLI-1 fusion gene. *Oncogene*. 1995;10:423–431.
68. Janknecht R, Nordheim A. Gene regulation by Ets proteins. *Biochim Biophys Acta*. 1993; 1155:346–356.
69. Huang HY, Illei PB, Zhao Z, et al. Ewing sarcomas with p53 mutation or p16/p14ARF homozygous deletion: a highly lethal subset associated with poor chemoresponse. *J Clin Oncol*. 2005;23:548–558.
70. Zucman J, Melot T, Desmaze C, et al. Combinatorial generation of variable fusion proteins in the Ewing family of tumors. *EMBO J*. 1993;12:4481–4487.
71. Jeon IS, Davis JN, Braun BS, et al. A variant Ewing's sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1. *Oncogene*. 1995;10:1229–1234.
72. Kaneko Y, Yoshida K, Handa M, et al. Fusion of an ETS-family gene, EIAF, to EWS by t(17;22)(q12;q12) chromosome translocation in an undifferentiated sarcoma of infancy. *Genes Chromosomes Cancer*. 1996;15:115–121.
73. Peter M, Couturier J, Pacquement H, et al. A new member of the ETS family fused to EWS in Ewing tumors. *Oncogene*. 1997;14:1159–1164.

74. Torchia EC, Jaishankar S, Baker SJ. Ewing tumor fusion proteins block the differentiation of pluripotent marrow stromal cells. *Cancer Res*. 2003;63:3464–3468.
75. Gonzalez I, Vicent S, de Alava E, Lecanda F. EWS/FLI-1 oncoprotein subtypes impose different requirements for transformation and metastatic activity in a murine model. *J Mol Med*. 2007;85:1015–1029.
76. Gershon TR, Oppenheimer O, Chin SS, Gerald WL. Temporally regulated neural crest transcription factors distinguish neuroectodermal tumors of varying malignancy and differentiation. *Neoplasia*. 2005;7:575–584.
77. Teitell MA, Thompson AD, Sorensen PH, Shimada H, Triche TJ, Denny CT. EWS/ETS fusion genes induce epithelial and neuroectodermal differentiation in NIH 3T3 fibroblasts. *Lab Invest*. 1999;79:1535–1543.
78. Rorie CJ, Thomas VD, Chen P, Pierce HH, O'Bryan JP, Weissman BE. The Ews/Fli-1 fusion gene switches the differentiation program of neuroblastomas to Ewing sarcoma/peripheral primitive neuroectodermal tumors. *Cancer Res*. 2004;64:1266–1277.
79. Hu-Lieskovan S, Zhang J, Wu L, Shimada H, Schofield DE, Triche TJ. EWS-FLI1 fusion protein up-regulates critical genes in neural crest development and is responsible for the observed phenotype of Ewing's family of tumors. *Cancer Res*. 2005;65:4633–4644.
80. Deneen B, Denny CT. Loss of p16 pathways stabilizes EWS/FLI1 expression and complements EWS/FLI1 mediated transformation. *Oncogene*. 2001;20:6731–6741.
81. Castillero-Trejo Y, Eliazer S, Xiang L, Richardson JA, Ilaria RL, Jr. Expression of the EWS/FLI-1 oncogene in murine primary bone-derived cells results in EWS/FLI-1-dependent, Ewing sarcoma-like tumors. *Cancer Res*. 2005;65:8698–8705.
82. Riggi N, Cironi L, Provero P, et al. Development of Ewing's sarcoma from primary bone marrow-derived mesenchymal progenitor cells. *Cancer Res*. 2005;65:11459–11468.
83. Tolar J, Nauta AJ, Osborn MJ, et al. Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells*. 2007;25:371–379.
84. Rangarajan A, Hong SJ, Gifford A, Weinberg RA. Species- and cell type-specific requirements for cellular transformation. *Cancer Cell*. 2004;6:171–183.
85. Riggi N, Suva ML, Suva D, et al. EWS-FLI-1 expression triggers a Ewing's sarcoma initiation program in primary human mesenchymal stem cells. *Cancer Res*. 2008;68:2176–2185.
86. Tirode F, Laud-Duval K, Prieur A, Delorme B, Charbord P, Delattre O. Mesenchymal stem cell features of Ewing tumors. *Cancer Cell*. 2007;11:421–429.
87. Szuhai K, Ijszenga M, Tanke HJ, Rosenberg C, Hogendoorn PC. Molecular cytogenetic characterization of four previously established and two newly established Ewing sarcoma cell lines. *Cancer Genet Cytogenet*. 2006;166:173–179.
88. Kovar H, Jug G, Aryee DN, et al. Among genes involved in the RB dependent cell cycle regulatory cascade, the p16 tumor suppressor gene is frequently lost in the Ewing family of tumors. Oncogene. 1997;15:2225–2232.
89. Tsuchiya T, Sekine K, Hinohara S, Namiki T, Nobori T, Kaneko Y. Analysis of the p16INK4, p14ARF, p15, TP53, and MDM2 genes and their prognostic implications in osteosarcoma and Ewing sarcoma. Cancer Genet Cytogenet. 2000;120:91–98.
90. Lopez-Guerrero JA, Pellin A, Noguera R, Carda C, Llombart-Bosch A. Molecular analysis of the 9p21 locus and p53 genes in Ewing family tumors. Lab Invest. 2001;81:803–814.
91. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3:730–737.
92. Jamieson CH, Weissman IL, Passegue E. Chronic versus acute myelogenous leukemia: a question of self-renewal. Cancer Cell. 2004;6:531–533.
93. Smith R, Owen LA, Trem DJ, et al. Expression profiling of EWS/FLI identifies NKX2.2 as a critical target gene in Ewing's sarcoma. Cancer Cell. 2006;9:405–416.
94. Owen LA, Kowalewski AA, Lessnick SL. EWS/FLI mediates transcriptional repression via NKX2.2 during oncogenic transformation in Ewing's sarcoma. PLoS ONE. 2008;3:e1965.

95. Fukuma M, Okita H, Hata J, Umezawa A. Upregulation of Id2, an oncogenic helix-loop-helix protein, is mediated by the chimeric EWS/ets protein in Ewing sarcoma. *Oncogene*. 2003;22:1–9.
96. Nishimori H, Sasaki Y, Yoshida K, et al. The Id2 gene is a novel target of transcriptional activation by EWS-ETS fusion proteins in Ewing family tumors. *Oncogene*. 2002;21:8302–8309.
97. Zwerner JP, May WA. PDGF-C is an EWS/FLI induced transforming growth factor in Ewing family tumors. *Oncogene*. 2001;20:626–633.
98. Matsumoto Y, Tanaka K, Nakatani F, Matsunobu T, Matsuda S, Iwamoto Y. Downregulation and forced expression of EWS-Fli1 fusion gene results in changes in the expression of G(1) regulatory genes. *Br J Cancer*. 2001;84:768–775.
99. Wai DH, Schaefer KL, Schramm A, et al. Expression analysis of pediatric solid tumor cell lines using oligonucleotide microarrays. *Int J Oncol*. 2002;20:441–451.
100. Dauphinot L, De Oliveira C, Melot T, et al. Analysis of the expression of cell cycle regulators in Ewing cell lines: EWS-FLI-1 modulates p57KIP2and c-Myc expression. *Oncogene*. 2001;20:3258–3265.
101. Bailly RA, Bosselut R, Zucman J, et al. DNA-binding and transcriptional activation properties of the EWS-FLI-1 fusion protein resulting from the t(11;22) translocation in Ewing sarcoma. *Mol Cell Biol*. 1994;14:3230–3241.
102. Takahashi A, Higashino F, Aoyagi M, et al. EWS/ETS fusions activate telomerase in Ewing's tumors. *Cancer Res*. 2003;63:8338–8344.
103. Nakatani F, Tanaka K, Sakimura R, et al. Identification of p21WAF1/CIP1 as a direct target of EWS-Fli1 oncogenic fusion protein. *J Biol Chem*. 2003;278:15105–15115.
104. Hahm KB. Repression of the gene encoding the TGF-beta type II receptor is a major target of the EWS-FLI1 oncoprotein. *Nat Genet*. 1999;23:481.
105. Im YH, Kim HT, Lee C, et al. EWS-FLI1, EWS-ERG, and EWS-ETV1 oncoproteins of Ewing tumor family all suppress transcription of transforming growth factor beta type II receptor gene. *Cancer Res*. 2000;60:1536–1540.
106. Prieur A, Tirode F, Cohen P, Delattre O. EWS/FLI-1 silencing and gene profiling of Ewing cells reveal downstream oncogenic pathways and a crucial role for repression of insulin-like growth factor binding protein 3. *Mol Cell Biol*. 2004;24:7275–7283.
107. Scotlandi K, Benini S, Nanni P, et al. Blockage of insulin-like growth factor-I receptor inhibits the growth of Ewing's sarcoma in athymic mice. *Cancer Res*. 1998;58:4127–4131.
108. Scotlandi K, Avnet S, Benini S, et al. Expression of an IGF-I receptor dominant negative mutant induces apoptosis, inhibits tumorigenesis and enhances chemosensitivity in Ewing's sarcoma cells. *Int J Cancer*. 2002;101:11–16.
109. Scotlandi K, Maini C, Manara MC, et al. Effectiveness of insulin-like growth factor I receptor antisense strategy against Ewing's sarcoma cells. *Cancer Gene Ther*. 2002;9:296–307.
110. Manara MC, Landuzzi L, Nanni P, et al. Preclinical in vivo study of new insulin-like growth factor-I receptor-specific inhibitor in Ewing's sarcoma. *Clin Cancer Res*. 2007;13:1322–1330.
111. Kinsey M, Smith R, Lessnick SL. NR0B1 is required for the oncogenic phenotype mediated by EWS/FLI in Ewing's sarcoma. *Mol Cancer Res*. 2006;4:851–859.
112. Garcia-Aragoncillo E, Carrillo J, Lalli E, et al. DAX1, a direct target of EWS/FLI1 oncoprotein, is a principal regulator of cell-cycle progression in Ewing's tumor cells. *Oncogene*. 2008.
113. Mendiola M, Carrillo J, Garcia E, et al. The orphan nuclear receptor DAX1 is up-regulated by the EWS/FLI1 oncoprotein and is highly expressed in Ewing tumors. *Int J Cancer*. 2006;118:1381–1389.

http://www.springer.com/978-1-84882-129-3

Pediatric Bone Sarcomas
Epiphysiolysis before excision
Cañadell, J.; San-Julian, M. (Eds.)
2009, XIV, 152 p., Hardcover
ISBN: 978-1-84882-129-3