

Immunophenotypic Profile of Biomarkers Related to Anti-apoptotic and Neural Development Pathways in the Ewing's Family of Tumors (EFT) and their Therapeutic Implications

S. NAVARRO¹, P. GIRAUDO¹, A.I. KARSELADZE², A. SMIRNOV², N. PETROVICHEV²,
N. SAVELOV², I. ALVARADO-CABRERO³ and A. LLOMBART-BOSCH¹

¹Department of Pathology, Medical School, University of Valencia, Spain;

²N.N. Blockhin Cancer Research Center, Moscow, Russia;

³Department of Pathology, Oncologic Hospital CMN Siglo XXI, México DF, México

Abstract. *Background:* Ewing's family of tumors (EFT) comprises a broad spectrum of tumors composed of primitive committed cells with neuroectodermal capacity. The degree of neural differentiation within EFT, as measured with morphological features and expression of neural markers, delimits two members: Ewing's sarcoma (ES) and peripheral primitive neuroectodermal tumor (pPNET). Molecules such as c-kit and its ligand (Stem cell factor, SCF), CD95 (FAS), CD95L (FASL), IGF-IR, protect EFT cells from apoptosis, whereas c-erb-B2, erythropoietin (EPO) and its receptor (EPO-R) participate in the maturation of primitive committed neuroectodermal cells and in the normal embryonal brain development. The aim of the present study was to analyse the expression of these molecules in paraffin-embedded material from a series of EFT. *Materials and Methods:* Forty-five cases of EFT (23 typical ES, 4 atypical and 18 pPNET) were analysed following the immunohistochemical LSAB method, with antigen retrieval heating using an autoclave, citrate buffer pH 6.0 and the following primary antibodies: FAS (APO-CD 95), FAS-L, c-kit, SCF, IGF-IR and c-erbB2. The expression was evaluated independently by three of the authors and the final score (0 to 3+) was based on the intensity and percentage of positively stained cells. In a second cooperative analysis, tissues from 30 cases of EFT (15 typical, 3 atypical and 12 PNET) were immunostained with EPO and EPO-R. *Results:* High expression of c-kit/SCF (2+, 3+) was detected in 28/45 cases of EFT (62.2%), whereas FAS-FAS-L and IGF-IR were observed in 16/45 (37.7%) and 9/45 (20%), respectively. Regarding the

neuroectodermal pathway, membranous and cytoplasmic expression of c-erb-B2 was observed in 9/45 (20%) EFT, regardless of the morphological and immunohistochemical expression of conventional neural markers. High expression of EPO and EPO-R was observed in 20/30 EFT (66.6%). *Conclusion:* C-kit/SCF and EPO/EPO-R seem to participate in the pathway of anti-apoptotic and proliferative advantage, while c-erb-B2 does not play an important role in the neuroectodermal differentiation pathway in EFT cells.

The Ewing family of tumors (EFT) includes Ewing's sarcoma, the most common malignant bone tumor occurring in children and young adults, and peripheral primitive neuroectodermal tumor which is the second most common soft tissue malignancy in childhood, accounting for 20% of sarcomas. This family of tumors is defined genetically by specific chromosomal translocations resulting in fusion involving the EWS gene with members of the ETS family of transcription factors. From the diagnostic point of view, these tumors express a complex immunophenotype comprised of markers such as CD99 (MIC2, HBA71), Fli 1, NSE, HNK-1 and other neural markers. Despite the use of aggressive multimodal approaches, these tumors present a disappointingly low survival rate, and innovative and more effective treatment methods are needed.

The stem cell factor (SCF)/c-kit tyrosine kinase receptor pathway has been considered important for tumor growth and progression in several human neoplasms, furthermore the therapeutic use of tyrosine kinase inhibitors has proven very useful in tumors such as gastrointestinal stromal tumors (GIST) or chronic myelogenous leukemia (1). Moreover, it is known that c-kit/SCF are expressed in EFT and that SCF is capable of protecting tumor cells against apoptosis (2).

The c-erb-B2/ HER-2/neu is a key component of a complex signaling network, playing a critical role in the regulation of tissue growth, differentiation and neural development (3). Immunotherapy with trastuzumab, an anti

Correspondence to: Professor A. Llombart-Bosch, Department of Pathology, University of Valencia, Medical School, Avda Blasco Ibáñez, 17, 46010 Valencia. Spain. Tel: +34 963 864146, Fax: +34 963 864173, e-mail: antonio.llombart@uv.es

Key Words: c-Kit, c-erb-B2, stem cell factor, FAS, FAS ligand, EPO, EPO-R, Ewing's sarcoma, therapeutic targets.

Table I. List of antibodies used in the study.

Antibody	Type	Source	Dilution	A. retrieval
C-Kit	Polyclonal	Dako (Denmark)	1/50	Yes
Stem cell factor	Polyclonal (H-189)	S. Cruz Biotech. (USA)	1/100	Yes
FAS	Polyclonal (C-20)	S. Cruz Biotech. (USA)	1/100	Yes
FAS-L	Polyclonal (N-20)	S. Cruz Biotech. (USA)	1/100	Yes
IGFR-I	Polyclonal (H-78)	S. Cruz Biotech. (USA)	1/100	Yes
C-erbB ₂	Monoclonal(CB-11)	Novocastra (UK)	1/100	Yes
EPO	Polyclonal (N-19)	S. Cruz Biotech (USA)	1/50	Yes
EPO-R	Polyclonal (H-194)	S. Cruz Biotech (USA)	1/50	Yes

c-erb-B2 monoclonal antibody, has proven useful in patients with metastatic breast carcinoma. Controversy exists regarding the overexpression of c-erb-B2 in EFT (4, 5) and no conclusive results have been reported.

Another hypothetical target could be the insulin-like growth factor I receptor that is expressed in EFT (6, 7) and could be applied to novel therapeutic approaches.

FAS-FASL interactions play a key role in the regulation of apoptosis within the immune system. Expression of FAS-FASL has been reported *in vitro* and *in vivo* in EFT, suggesting that these tumors are potential targets for immunotherapy (8-10).

Erythropoietin (EPO), usually considered a red blood cell-restricted cytokine, plays a broader role in non-hematopoietic tissues. Recent publications demonstrated an important role of EPO and its receptor (EPO-R) in the normal embryonal brain development (11, 12). Expression of EPO and EPO-R has been demonstrated in neurons of the hippocampus, cortex and midbrain areas of the brain where it promotes survival in the presence of hypoxia.

Regarding neoplastic pathology, high levels of EPO and EPO-R expression have been demonstrated in adult tumors of the female reproductive system such as breast (primary tumors and cell lines), uterus and ovary (13-15).

More than 50% of pediatric solid neoplasms are of neural origin and/or have neural differentiation. This is the case in EFT, retinoblastoma, neuroblastoma and tumors of the central nervous system.

Recently, Batra *et al.* (16) described a high expression of EPO and EPO-R in cell lines as well as in primary tumors of diverse lineages, especially in neural tumors. Moreover, the authors showed that EPO production in cell lines was induced by hypoxia and that EPO promoted an antiapoptotic and angiogenic response in these cell lines.

Controversy exists regarding the use of EPO in the management of pediatric tumors in order to avoid excess of red blood cell transfusions (17-19). Thus, the use of EPO antagonists or agents that induce a blockade of EPO-R signaling have to be strictly evaluated in order to be incorporated into therapeutic trials.

Our objective was to analyse the expression *in vivo* of such molecules in EFT, using archival paraffin-embedded material, in order to investigate the hypothetical use of inhibitors of these molecules as therapeutic targets and also to relate such expression to the degree of neural differentiation in EFT.

Materials and Methods

Forty-five cases of EFT (23 typical Ewing's sarcoma, 4 atypical and 18 PNET classified by conventional immunohistochemistry (CD-99, Fli-1 and a wide panel of neural markers including HNK-1, NSE, S-100, Synaptophysin, PGP9.5 and neurofilaments) were analysed following the LSAB-2 method, with antigen retrieval heating using an autoclave and citrate buffer pH-6.0. In a second cooperative analysis, 30 cases of EFT (15 typical, 4 atypical and 12 PNET) were immunostained for EPO and EPO-R.

The list of antibodies as well as their characteristics are summarised in Table I.

The expression was evaluated independently by three authors (SN, ALLB, AIK). The immunostaining was scored using a 3-tier system for all the antibodies as follows: Negative 0%, mild 1-25% (+), moderate 26-50% (++) and intense 51% and above (+++). The agreement of staining intensity scoring between the three observers was recorded and in cases of disagreement, intensity was determined by consensus. Moreover, a second evaluation for intensity was performed according to the guidelines of The Quality Control Programme for Immunohistochemistry from the Spanish Society of Pathology as follows: 0 no staining, 1 low intensity of staining, 2 moderate intensity, and 3 excellent intensity.

Results

The immunohistochemical results are summarised in Tables II and III.

High expression of c-kit/SCF (2+, 3+) was detected in 28/45 cases of EFT (62.2%). The staining pattern was mainly membranous, but in 30% of the cases a cytoplasmic expression of c-kit was evident. High expression of c-kit (3+) was only observed in 6 cases (13.3%): 4 cases were typified as typical Ewing's sarcoma, and 2 cases as EFT with neural phenotype. The immunophenotypic analysis of stem cell factor (SCF), revealed almost the same staining pattern, and moreover, the

Table II. Immunohistochemical results.

Case	Diagnosis	Antibody					
		C-KIT	SCF	FAS	FAS-L	IGFR-I	C-ERB2
RU-1	Ewing - T	++ (2)	++ (2)	++ (1)	+ (1)	Ø (0)	+(1)
RU-7	Ewing - N	++ (3)	++ (2)	++ (1)	++ (1)	+ (1)	+++ (3)
RU-9	Ewing - N	Ø (0)	Ø (0)	+ (1)	+ (1)	+ (1)	+(1)
RU-10	Ewing - N	Ø (0)	Ø (0)	+ (1)	+ (1)	Ø(0)	Ø(0)
RU-11	Ewing - T	+ (2)	++ (2)	+ (1)	+ (1)	+	Ø(0)
RU-12	Ewing - T	Ø (0)	Ø (0)	+ (1)	+ (1)	Ø(0)	+(1)
RU-14	Ewing - T	+ (2)	+ (1)	Ø (0)	Ø (0)	Ø(0)	Ø(0)
RU-15	Ewing - T	++ (3)	N/V	Ø (0)	Ø (0)	Ø(0)	Ø(0)
RU-16	Ewing - T	Ø (0)	+ (2)	++ (1)	+ (1)	+(1)	Ø(0)
RU-17	Ewing - N	+++ (3)	++ (3)	++ (1)	Ø (0)	++(1)	Ø(0)
RU-18	Ewing - N	++ (3)	++ (3)	Ø (0)	+ (1)	+(1)	Ø(0)
RU-19	Ewing - A	++(3)	++ (3)	+ (1)	Ø (0)	Ø(0)	Ø(0)
RU-20	Ewing - N	++ (3)	++ (3)	++ (1)	++ (1)	++(1)	++(1)
RU-21	Ewing - T	++ (3)	++ (3)	++ (1)	++ (1)	++(1)	++(3)
RU-22	Ewing - T	+++ (3)	++ + (3)	++ (1)	++ (1)	+(1)	Ø(0)
RU-23	Ewing - N	+++ (3)	++ + (3)	+ (1)	++ (1)	+(1)	++ + (3)
RU-24	Ewing - T	+++ (3)	++ (3)	++ (1)	+ (1)	+(1)	++(1)
RU-25	Ewing - T	+ (1)	+ (1)	+ (1)	+ (1)	+(1)	+ (1)
RU-26	Ewing - T	+++ (3)	+++ (3)	++ (1)	++ (1)	+(1)	+ (1)
RU-27	Ewing - T	++ (2)	++ (3)	+ (1)	+ (1)	Ø(0)	++(1)
RU-28	Ewing - T	++ (2)	++ (3)	+ (1)	+ (1)	Ø(0)	++(1)
RU-42	Ewing - T	++ (2)	+ (1)	Ø (0)	Ø (0)	Ø(0)	Ø(0)
RU-43	Ewing - T	+ (2)	Ø (0)	Ø (0)	Ø (0)	Ø(0)	Ø(0)
RU-44	Ewing - T	++ (3)	++ (2)	Ø (0)	Ø (0)	Ø(0)	Ø(0)
RU-45	Ewing - N	++ (3)	Ø (0)	Ø (0)	Ø (0)	Ø(0)	Ø(0)
RU-47	Ewing - T	Ø (0)	Ø (0)	Ø (0)	Ø (0)	Ø(0)	Ø(0)
RU-50	Ewing - N	++ (2)	++ (2)	+ (1)	Ø (0)	Ø(0)	+(1)
RU-54	Ewing - A	Ø (0)	+ (2)	++ (1)	++ (1)	++(1)	++ + (3)
RU-58	Ewing - N	++ (2)	++ (2)	++ (1)	+ (1)	Ø(0)	+(1)
ME-1	Ewing - T	++ (2)	++ (2)	++ (1)	++ (1)	++(1)	Ø(0)
ME-4	Ewing - N	++ (2)	++ (2)	+++ (2)	++ (1)	+(1)	Ø(0)
ME-5	Ewing - N	++ (2)	+ (2)	Ø (0)	+ (1)	Ø(0)	Ø(0)
ME-6	Ewing - N	Ø (0)	Ø (0)	++ (1)	++ (1)	+(1)	+(1)
ME-7	Ewing - A	+ (1)	+ (2)	++ (1)	++ (1)	Ø(0)	+ (1)
ME-8	Ewing - T	+++ (3)	++ (2)	+ (1)	+ (1)	++(1)	+ (1)
ME-10	Ewing - T	++ (3)	++ (2)	++ (1)	++ (1)	+(1)	Ø(0)
ME-12	Ewing - N	+ (2)	+ (2)	+ (1)	+ (1)	Ø(0)	N/V
ME-13	Ewing - N	+ (2)	Ø (0)	+++ (3)	++ (1)	+(1)	+ (1)
ME-16	Ewing - T	++ (3)	++ (2)	++ (1)	++ (1)	++(1)	+ (1)
ME-17	Ewing - T	++ (3)	N/V	+ (1)	Ø (0)	++(1)	Ø(0)
ME-19	Ewing - A	++ (3)	+ (1)	++ (1)	++ + (1)	++(1)	Ø(0)
U-10	Ewing - T	++ (3)	++ (2)	++ (1)	++ (1)	+(1)	Ø(0)
U-12	Ewing - N	+ (2)	+ (1)	+ (1)	+ (1)	Ø(0)	N/V
U-13	Ewing - N	+ (2)	Ø (0)	+++ (3)	++ + (1)	+(1)	+ (1)

Ewing-N: PNET; Ewing-A: atypical; Ewing-T: typical.

same cases that overexpressed c-kit, presented high expression of SCF with membranous and cytoplasmic staining. No differences in staining between diffuse undifferentiated areas of EFT typical, atypical and neuroectodermal, such as pseudorosettes, were found (Figure 1 A, B).

FAS-FASL and IGF-IR expression was observed in 16/45 (37.7%) and 9/45 (20%) of cases respectively. As occurred

with c-kit, the staining pattern was also membranous, although in several cases, some cytoplasmic impregnation was evident. The immunostaining was weaker than that observed with c-kit-SCF and only 6% of cases presented high expression (3+) of FAS/FASL (Figure 1 C-E).

Membranous and cytoplasmic expression of c-erbB2 was observed in 9/45 (20%) of EFT, regardless of the

Table III. Immunohistochemical results of Ewing family tumors.

Ewing typical	EPO	EPO-R	Ewing atypical (A) and neural (N-PNET)	EPO	EPO-R
EWS-1	+(1)	+(1)	EWS-16 N	+(1)	+(1)
EWS-2	Ø(0)	Ø(0)	EWS-17 A	Ø(0)	Ø(0)
EWS-3	+(1)	Ø(0)	EWS-18 N	++(1)	+++(2)
EWS-4	++(3)	+(1)	EWS-19 N	+(1)	+(1)
EWS-5	+++(3)	+(1)	EWS-20 A	+(1)	+(1)
EWS-6	Ø(0)	Ø(0)	EWS-21 N	Ø(0)	Ø(0)
EWS-7	+(1)	+(1)	EWS-22 N	Ø(0)	Ø(0)
EWS-8	Ø(0)	Ø(0)	EWS-23 A	+(1)	+(1)
EWS-9	Ø(0)	Ø(0)	EWS-24 N	+(1)	+(1)
EWS-10	+(1)	+(1)	EWS-25 N	Ø(0)	Ø(0)
EWS-11	+(1)	+(1)	EWS-26 N	+(1)	++(2)
EWS-12	++(1)	+(1)	EWS-27 N	+(1)	+(1)
EWS-13	+(1)	+(1)	EWS-28 N	Ø(0)	Ø(0)
EWS-14	+(1)	+(1)	EWS-29 N	+(1)	+(1)
EWS-15	Ø(0)	Ø(0)	EWS-30 N	++(2)	+(1)

morphological and immunohistochemical expression of conventional neural markers (Figure 1 F). In three of the positive cases, granular cytoplasmic immunostaining was notable.

Diffuse cytoplasmic immunostaining was observed for EPO, whereas the immunoprofile of EPO-R was mainly membranous-cytoplasmic. Coexpression of both biomarkers was evident in 20/30 EFT, with no correlation with the degree of neuroectodermal differentiation (Figure 2 A, B).

Discussion

The EFT represents a heterogeneous group of small round cell tumors with diverse anatomical locations, affecting not only bone and soft tissue, but also, though less frequently, solid organs. These tumors, independent of their anatomical locations, belong to the category of peripheral primitive neuroectodermal tumors, expressing a variable degree of neural maturation capacity at a phenotypic level, but based on a common histogenetic origin (11). Due to their complexity at the phenotypic level (morphologically and immunocytochemically), and to the existence of a well-defined genotype, they have become a model system for diagnostic, prognostic and therapeutic purposes.

Therapy for EFT patients is based mainly on RT and chemotherapy protocols, with variable results, survival ranging between 30% and 50% of cases. Several studies have attributed antiapoptotic functions to the associated fusion oncproteins. Interfering with these tumor-specific products may affect the survival and growth of tumor cells. Molecular therapies directed against the novel fusion gene product could be incorporated into new strategies (15, 17, 18).

The need for innovative, more effective treatment modalities for EFT prompted the study of signaling pathways that induce a proliferative advantage for tumor cells and protect them from apoptosis. One of the well-studied molecules is the c-kit proto-oncogene that encodes a 145-165 kDa membrane-bound glycoprotein of the tyrosine-kinase family and its ligand SCF that configures a system playing a crucial role in normal melanogenesis, gonadogenesis and hematopoiesis. In human tumors such as chronic myeloid leukemia and GIST, mutations of the c-kit gene as well as the overexpression of the c-kit protein allows the use of tyrosine kinase inhibitors, such as ST1571, as therapeutic targets, with satisfactory results (19, 20). C-kit/SCF expression has been described in EFT *in vitro* (1) as well as *in vivo*, with different expression results, ranging from 20% of cases (21) to 71% of cases (2). In the afore-mentioned reports, the results indicated that SCF/c-kit induces growth advantage, inhibiting apoptosis, either *via* autocrine stimulation by SCF, or SCF stabilization of a constitutively active c-kit receptor. In the present study, we detected high expression of c-kit/SCF in almost 63% of cases. High expression of c-kit and SCF (2+, 3+) was seen in the same cases, suggesting an autocrine loop. The therapeutic relevance of c-kit expression *per se*, without mutations of the gene, is controversial and mutational analysis of c-kit should be performed if ST1571 is to be used. Currently available therapy targeted against receptor tyrosine kinases such as c-kit are only active against mutant protein and mutations of c-kit have not been demonstrated in EFT. A recent study (22) demonstrated a limited *in vitro* therapeutic activity of ST1571 in a series of EFT cell lines, indicating that the putative aberrant signaling provided by c-kit overexpression may not be essential for EFT development. In the same study, the

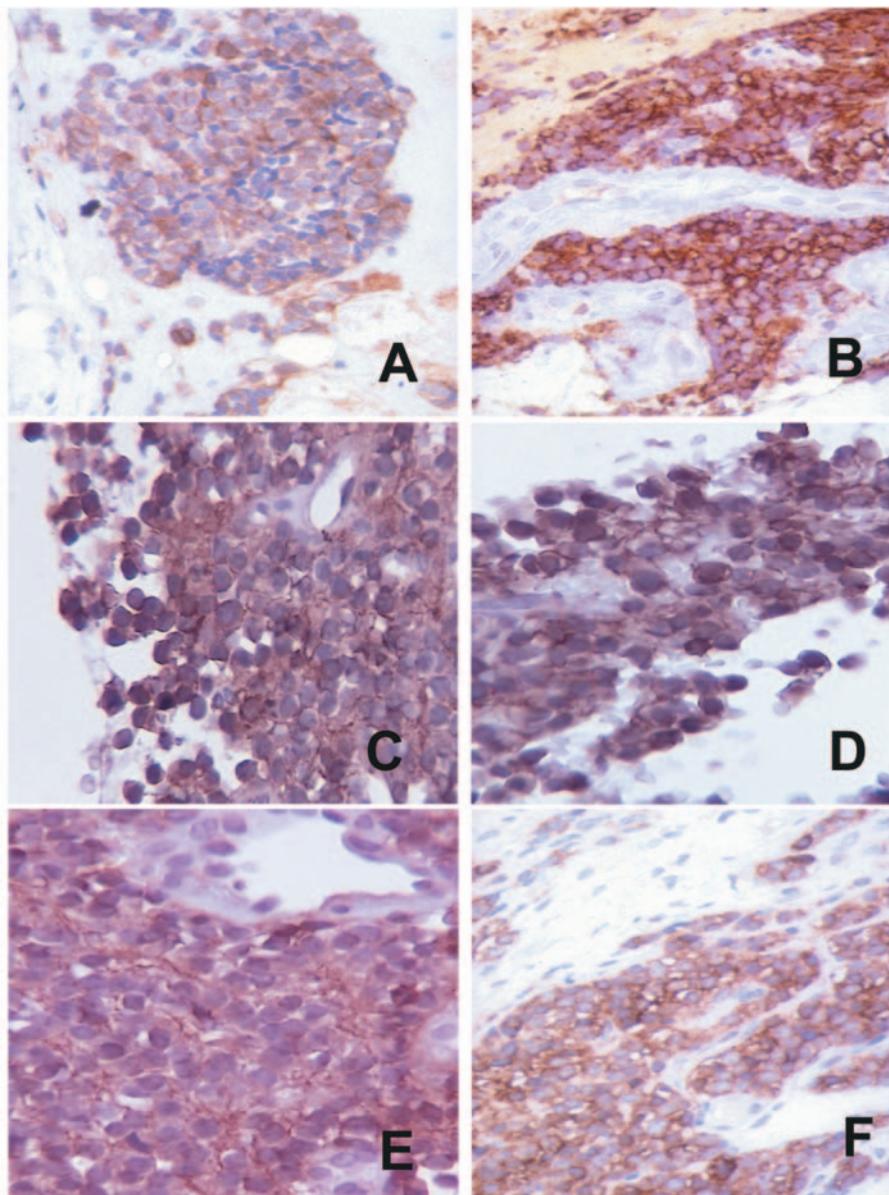


Figure 1. Immunohistochemical results. A) c-Kit expression in Ewing's sarcoma with membranous immunostaining. B) Ewing's sarcoma showing membranous expression of stem cell factor. C) FAS membranous staining in a case of pPNET. D) FASL expression in pPNET. E) Membranous staining of ILGF-IR in Ewing's sarcoma. F) Cytoplasmic and membranous expression of c-erbB2 in Ewing's sarcoma. (ABC peroxidase with antigen retrieval, $\times 40$).

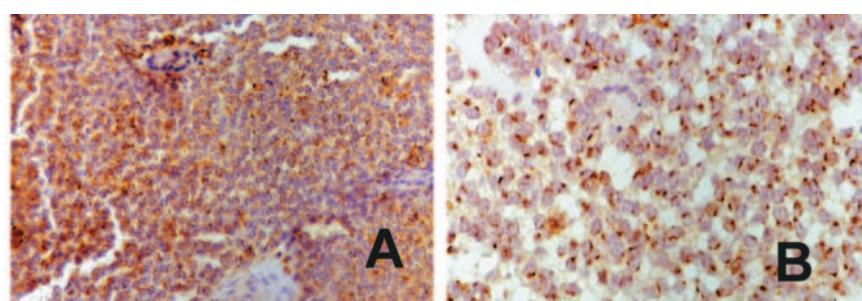


Figure 2. Immunohistochemical results. A) EPO expression in Ewing's sarcoma. B) EPO-R immunostaining in Ewing's sarcoma. (ABC peroxidase with antigen retrieval, $\times 25$).

authors also detected an inhibition of cell growth when the drug was administered in combination with standard therapy such as doxorubicin (22).

Another autocrine system analysed in EFT is that involving the insulin-like growth factor I receptor (IGF-IR). Scotlandi *et al.* (6) demonstrated the relevance of an autocrine loop mediated by the IGF-IR that is crucial for the survival and proliferation of EFT cells *in vivo*, suggesting the use of IGF-IR-blocking monoclonal antibodies for therapeutic strategies. In our immunohistochemical study, we report expression of IGF-IR in only 20% of cases with no differences based upon major neuroectodermal differentiation.

Another interesting system is that of FAS-FASL, which plays a central role in the regulation of the immune response. FAS ligand expression by tumors has been implicated in the abrogation of the host antitumor response by killing of FAS-positive effector lymphocytes. Expression of FAS-FASL has been documented in EFT (8, 10) and, moreover, metastatic tumors presented higher expression of FASL suggesting an association with a metastatic phenotype, proposing its use as a potential target for immunotherapy (9). In our cases, over a third (37.7%) of tumors with or without neural differentiation, presented expression of both markers, a fact that may be of interest from the therapeutic point of view.

c-erbB2(HER2) is a key component of the signaling network that plays a critical role in the regulation of neural development (4). Expression of c-erbB2 in sarcomas has been reported, mainly in the epithelial glandular component of synovial sarcomas (23); such expression has been observed in EFT cell lines (5), but not *in vivo* (3). As is well known, immunotherapy with monoclonal antibodies against HER2 are in use in several protocols for advanced breast carcinoma (24).

In the present study, membranous and cytoplasmic expression of c-erbB2 was observed in 20% of cases of EFT, regardless of the morphological and immunohistochemical expression of conventional neural markers. Here again it seems that an in-depth analysis would be valuable in view of the new therapeutic assays becoming available. Regarding neuroectodermal maturation, the present observations could not provide a distinction between the two variants of EFT.

EPO and EPO-R expression has been demonstrated in primary neoplasms and tumor cell lines such as prostate cancer (25). Moreover, such expression has been also described in pediatric tumors (16), especially in Wilms' tumors, Ewing's sarcoma, neuroblastoma and embryonal rhabdomyosarcoma. In our study, we found a high expression of EPO and EPO-R in the EFT cases, suggesting an autocrine cell signaling pathway. This expression was previously observed in paraffin sections from neuroblastoma (in relation to neural differentiation/maturation), as well as in alveolar and embryonal rhabdomyosarcoma (26).

The present results add to the controversy regarding the use of EPO in the management of pediatric tumors with intensive chemotherapy regimes in order to avoid an excess of red blood cell transfusions. On the other hand, the use of EPO antagonists or agents that induce a blockade of EPO-R signaling should be evaluated in order to incorporate them as a therapeutic modality in EFT.

Conclusion

Our results suggest that c-kit/SCF expression could play a role in the antiapoptotic pathway and confer proliferative advantage in EFT, indicating the possibility of using these molecules as therapeutic targets. Secondly, other molecules such as IGF-IR and FAS-FASL, which are expressed at lower levels in EFT, could also be considered as hypothetical therapeutic targets. Finally, our results indicate that c-erbB2 expression does not correlate with neuroectodermal differentiation in EFT, but its detection in some cases suggests the use of monoclonal antibodies as an alternative therapy, especially in those tumors that overexpress the c-erbB2 oncoprotein. Further studies are necessary to confirm present results and are underway with a large number of cases from several centers involved in a recently initiated European cooperative project.

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