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Expression of insulin-like growth factor system components in Ewing's sarcoma and their association with survival ☆

Katia Scotlandi [a,b,*], *Maria Cristina Manara* [a], *Massimo Serra* [a], *Maria Teresa Marino* [a], *Selena Ventura* [a], *Cecilia Garofalo* [a], *Marco Alberghini* [c], *Giovanna Magagnoli* [a], *Stefano Ferrari* [d], *Jose Antonio Lopez-Guerrero* [e], *Antonio Llombard-Bosch* [f], *Piero Picci* [a]

[a] *Laboratory of Experimental Oncology, Orthopaedic Institute Rizzoli, Bologna, Italy*
[b] *Centre for the Development of Biomolecular Therapies, Orthopaedic Institute Rizzoli, Bologna, Italy*
[c] *Department of Pathology, Orthopaedic Institute Rizzoli, Bologna, Italy*
[d] *Chemotherapy Service, Orthopaedic Institute Rizzoli, Bologna, Italy*
[e] *Laboratory of Molecular Biology, Oncologic Institute Valenciano, Valencia, Spain*
[f] *Department of Pathology, University of Valencia, Spain*

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ABSTRACT

Aims: The role of IGF system in the pathogenesis of Ewing's sarcoma (EWS) is well-documented. However, still little information is available about the value of IGF system components as indicators of prognosis. Understanding the clinical role for IGF system in EWS patients may be important because different subtypes of patients have distinct outcome and may require different treatment protocol. We evaluated the expression of insulin-like growth factor (IGF)-receptor (IGF-IR), insulin receptor (IR), IGF-I and some major intracellular mediators (IRS1, p-ERK) in specimens from EWS patients with primary localised untreated tumours.

Patients and methods: 290 samples were used for immunohistochemistry studies; 57 samples for Real-Time PCR studies; and serum of 67 patients for ELISA.

Results: IGF-IR and IR are expressed in virtually all EWS tumours. Usually both the receptors are present in the same tumour but when one receptor is lacking the other one is always present. Evaluation of IGF-IR, IR and IGF-I with quantitative methods may discriminate differential levels of expression with influences on the patients' outcome. High expression of IGF-IR, IR and IGF-I mRNAs is significantly associated with more favourable clinical outcomes. Higher circulating levels of IGF-I also correlated with lower risk to disease progression and death.

Conclusions: Overall, our clinical data are in contrast with the assumption that higher amounts of IGF/IGF-IR are a surrogate for higher aggressiveness and indicate that in some cancers the transition to frank malignancy and poor treatment responsiveness seem to be associated with a reduction of IGF system activity.

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* *Corresponding author*: Address: CRS Development of Biomolecular Therapies, Laboratory of Experimental Oncology, Orthopaedic Institute Rizzoli, Bologna 40136, Italy. Tel.: +39 051 6366760; fax: +39 051 6366763.
E-mail address: katia.scotlandi@ior.it (K. Scotlandi).

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1. Introduction

Ewing's sarcoma (EWS) is a highly aggressive bone tumour that occurs predominantly in children and young adults. Patients with localised EWS at diagnosis show a survival rate of around 65% due to the adoption of multimodal approaches, including local control of the disease by surgery and/or radiotherapy and multidrug adjuvant chemotherapy.[1,2] However, improvements have been achieved by dose-intensification, therefore, paying the price of severe toxicity and high rate of life-threatening late events, such as secondary malignancies.[3,4] Survivors of sarcomas, together with those of brain tumours, had the lowest health quality of life scores[5] and high cure costs, due to limb-salvage procedure, prolonged dose-dense chemotherapy (around 1 year), development of chronic severe pathologies. Thus, even in the best situation of localised tumours at diagnosis there is the need of complementary biomolecular approaches that will help keep treatment-related toxicity to a minimum and ameliorate systemic-disease control.[6] Costs for cure optimisation is another very important aspect for tumours that mainly relies on public or no-profit investments. Thus, it is imperative to generate more individualised treatment regimens and define best combinations between targeted and conventional treatments. Discoveries in the last years have led to a better understanding of the mechanisms involved in the genesis of Ewing's sarcoma and allowed the identification of some biological targets (for a review see.[7,8] Particularly insulin-like growth factor-insulin receptor (IGF-IR) system attracted experimental and clinical interest. Its role in the pathogenesis of this tumour is well-documented,[9,10] and phase I and II clinical studies had substantially confirmed the therapeutic potentialities of IGF-IR targeting agents against a few EWS.[11] However, the value of IGF system components as indicators of EWS prognosis has not been explored.

The IGF system is composed of multiple receptors and ligands. There are three ligands (insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II) and insulin), four receptors, at least six high affinity binding proteins and binding protein proteases. Insulin and IGFs bind with high affinity to their cognate receptor and with lower affinity to the non-cognate receptor. Molecular details of the IGF system can be found in excellent reviews.[12,13] Since IGF-I was found to be the principal ligand produced by EWS cells,[14,15] we focused our attention on IGF-I, IGFBP-3, the major regulator of autocrine/paracrine IGFs, IGF-IR, IRS-1, one of the major intracellular mediators of IGF-IR/PI3K signalling, and p-Erk, which is involved in MAPK signalling. Evaluation of insulin receptor (IR) was also included. A role for insulin in EWS prognosis is biologically plausible, given that overexpression of IR isoform A (a foetal insulin receptor associated with enhanced mitogenic signalling) is often present in tumours, including sarcomas,[16] that insulin and IGF-I can weakly interact with each other's receptor,[12,16] and that IR is associated with innate and acquired resistance to anti-IGF-IR agents.[17,18] In this paper, we evaluated the mRNA and protein expression of these major components of IGF system in primary tumour biopsies and their influence on EWS prognosis. Circulating levels of IGF-I and IGFBP-3 in patients with EWS were also analysed.

2. Patients and methods

2.1. Patients

Tissue and serum samples were collected from the tissue bank of the Laboratory of Experimental Oncology, Rizzoli Institute. Histology was reviewed by a pathologist panel. Standard follow-up surveillance protocol was applied and clinical data updated. Adverse events were defined as recurrence of the tumour at any site or death during remission,

Table 1 – Clinicopathologic features of the 290 EWS patients evaluated for IGF system expression by immunohistochemistry.

	No.	%	Association with prognosis (EFS)[a]
Gender			
Female	113	39	0.42
Male	177	61	
Age			
⩽14	126	43	0.08
>14	164	57	
Location			
Extremity	166	57	0.09
Pelvis	75	26	
Other	49	17	
Diagnosis			
Ewing' sarcoma	191	67	0.06
PNET	41	14	
Athypical	56	19	
Surgery			
YES	176	62	0.003
NO	109	38	
Chemotherapy			
YES	279	96	0.01
NO	11	4	
Chemotherapy protocol			
EW-REN1-3	122	44	
ISG/SSGIII-IV	72	26	
REA1-2	85	30	0.01[b]
Necrosis[c]			
Total	36	28	0.002
Non-total	91	72	
EFS (Status)			
NED	158	56	
REL	123	44	
OVS (Status)			
Alive	145	52	
Dead	136	48	

Abbreviations: EWS, Ewing's sarcoma; PNET, primitive neuroectodermal tumours; NED, no evidence of disease; REL relapsed.

[a] EFS, event-free survival; similar results for over-all survival (OVS, not shown).

[b] REA1-2 associated with worse prognosis.

[c] Data available for 127 patients only out of the 176 that underwent surgery. Median follow-ups: 142 months; range 12–452 months.

Table 2 – Clinicopathologic features of EWS patients evaluated for IGF system expression by RT-PCR in tissue samples or by ELISA in serum.

Characteristics	RT-PCR ($n = 57$)			ELISA ($n = 67$)		
	No.	%	Association with prognosis (EFS)[a]	No.	%	Association with prognosis (EFS)[a]
Gender						
Female	21	37	0.30	23	34	0.63
male	36	63		44	67	
Age						
⩽14	19	33	0.22	31	46	0.74
>14	38	67		36	54	
Location						
Extremity	37	65	0.24	40	60	0.62
Pelvis	9	19		18	27	
other	11	16		9	13	
Diagnosis						
Ewing' sarcoma	46	81		57	85	0.36
PNET	10	17	0.36	6	9	
Athypical	1	2		4	6	
Surgery						
YES	46	81	0.78	55	82	0.02
NO	11	19		12	18	
Chemotherapy						
YES	57	100	nd	67	100	nd
NO	0	0		0	0	
Chemotherapy protocol						
EW-REN1-3	27	47	0.48	2	3	0.67
ISG/SSGIII-IV	30	53		65	97	
Necrosis[b]						
Total	13	29	0.07	19	35	0.008
Non-total	32	71		36	65	
EFS (Status)						
NED	20	35		42	63	
REL	37	65		25	37	
OVS (Status)						
Alive	29	51		53	79	
Dead	28	49		14	21	

Abbreviations: EWS, Ewing's sarcoma; PNET, primitive neuroectodermal tumours; NED, no evidence of disease; REL relapsed.

[a] EFS, event-free survival; similar results for over-all survival (OVS, not shown).

[b] Data available for the patients that underwent surgery. (Median follow-ups: 88 months; range 12–192, for the 57 cases analysed by RT-PCR; 48 months, range: 16–90 months for the 67 cases analysed by ELISA).

and event-free survival was calculated from the date of initial diagnosis. Median follow-ups were, respectively: 142 months; range 12–452 months, for the 290 cases analysed by immunohistochemistry; 88 months; range 12–192, for the 57 cases analysed by RT-PCR; 48 months, range: 16–90 months for the 67 cases analysed by ELISA). Clinical pathologic features of the different series of EWS patients included in the study are summarised in Tables 1 and 2. Sample sets were handled in a coded fashion. The ethical committee of the Rizzoli Institute approved the studies and informed consent was obtained.

2.2. RNA extraction and fusion type characterisation

Quality of the frozen specimens was checked by haematoxylin-and-eosin staining. Total RNAs were extracted by TRIzol extraction kit (Invitrogen Ltd., Paisley, United Kingdom) and 0.5 μg of total RNA for each sample was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For the identification of fusion transcripts, primers detecting different chimeric products were used.[19]

2.3. Real time PCR analysis

Pre-designed TaqMan probes and primers sets for genes were chosen (IRS1: Hs00178563_m1; IGF1R: Hs00181385_m1; IGFBP3: Hs00426287_m1; IGF1: Hs00153126_m1; GAPDH: Hs99999905_m1; CCND1: Hs00277039_m1) and Syber Green and primers sets (IR: AH002851.1) and three replicates per gene were considered. Samples were analysed using ABI Prism® 7900 Sequence Detection System (Applied Biosystems),

according to manufacturer's instructions. Expression levels of target genes were normalised to GAPDH and the relative quantification analysis was performed on the basis of the ΔΔCT method. cDNA from human bone marrow $CD34^+$ cells was used as calibrator for the comparative analysis.

2.4. Immunohistochemistry

Representative samples of paraffin-embedded EWS/PNET tumours, at least three cores per patients, were included in tissue microarray (TMA). Avidin–biotin–peroxidase procedure was used for immunostaining (Vector Laboratories, Burlingame, CA). Briefly, sections were treated sequentially with xylene and ethanol to remove paraffin. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in methanol for 30 min at room temperature. To ensure antigen retrieval, the tissue sections were pretreated with a citrate buffer solution [0.01 mol/L citric acid and 0.01 mol/L sodium citrate (pH 6.0)] in a microwave oven at 750 W for three cycles of 5 min. A further blocking step with normal horse serum was performed. TMA were then incubated ON at 4 °C with the following primary antibodies: anti-IRS1 antibody (Upstate-Millipore, Billerica, MA) diluted 1:30, anti p-ERK (Tyr202/Tyr204), (Covance, Princeton, NJ) diluted 1:10, anti-IR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:10 or anti-IGFIRβ antibody (Santa Cruz Biotechnology) diluted 1:50. Samples were classified on the basis of the positivity score as follows: 'low-expressors', when no staining or low positivity was observed (for IRS-1, less than 10% of positive cells, score 1; for IGF-IR or IR, when the intensity of the staining was scored as +/−, +−−); 'high-expressors', when a diffused immunostaining was present (for IRS-1: positive cells between 10% to 50% = score 2, or positive cells ⩾ 50%, score 3; for IGF-IR or IR: intensity of the staining scored as ++− or +++). Regarding p-ERK, tumours were simply defined as positive or negative, since the antibody recognises only the activated form of the signalling mediator.

2.5. ELISA evaluation of IGF-I and IGFBP-3 in EWS patients sera

Insulin Growth Factor-1 (IGF-1) ELISA (Biosource Europe S.A., Belgium) and Human IGFBP-3 Immunoassay (R&D Systems, Inc., Minneapolis, MN) were used respectively. The quantification protocols were performed accordingly to manufacturer's instructions.

2.6. Statistical analysis

Differences among means were analysed using Student's t-test or non-parametric Mann–Whitney rank sum test, when data were not normally distributed. Pearson's test was used for correlations. Kaplan–Meier and log-rank methods were used, respectively, to draw and evaluate the significance of survival curves in EWS patients. Cox's proportional-hazards regression analysis was used for multivariate analysis.

3. Results

3.1. Expression of IGF-IR, IRS-1 and p-Erk in EWS and their association with prognosis

We analysed the expression of IGF-IR, IR and two of their major intracellular mediators: IRS-1, an adaptor protein mainly connected with PI3K pathway, and p-ERK, a component of the MAPK pathway in primary localised untreated EWS tumours. IGF-IR was detectable at membrane level, IR immunostaining was observed either at the cell surface or in the cytoplasm, IRS-1 in the cytosol and nucleus of the cells, while p-ERK showed a prevalent cytoplasmic localisation. Expression of IGF-IR in primary localised tumours was detected in 272/290 samples, 94% of the samples. Among the positive cases, the great majority was classified as high-expressors (195/272, 72%). IR was found to be expressed in 95% of patients and 78% of them were classified as high-expressors (Fig. 1a). Tumours that were negative for IGF-IR, expressed IR and vice versa. In most cases both the receptors are present. High-expressors for IRS-1 were 66%. p-ERK was found to be activated in 39% of cases (Fig. 1a). Expression of IGF-IR was found to be statistically associated, case by case, with the expression of IRS-1 ($r = 0.45$, $p < 0.0001$, Pearson's correlation test) and with p-ERK ($r = 0.40$, $p = 0.002$, Pearson's correlation test), confirming the soundness of our findings. No significant correlation was instead observed between IR and the other components here examined.

Clinicopathological features of the 290 patients are reported in Table 1. Complete clinical information related to patients' outcome was available for 281 tumours (median follow-up 142 months; range 12–452 months). Considering clinical parameters, patients who underwent surgery and/or chemotherapy and patients who had a total necrosis after pre-operative chemotherapy showed a favourable prognosis (Table 1). Among the different chemotherapy protocols, adjuvant chemotherapy (protocols REA1-2) was associated with a worse prognosis. In contrast, no significant differences were observed in terms of survival for the two neo-adjuvant chemotherapeutic protocols EW-REN1-3 and ISG/SSGIII-IV (Table 1). For this reason, prognostic relevance of IGF-IR, IR, IRS-1 and p-ERK was analysed considering only the 194 cases that underwent EW-REN1-3 or ISG/SSGIII-IV. Associations with patient's disease-free or overall survival did not indicate a prognostic role for any of the three molecules here considered (Fig. 1b). Similar results were also obtained with the entire series of 290 patients (data not shown).

3.2. Prognostic value of IGF system gene expression in EWS

To obtain a more quantitative evaluation of the IGF system in EWS patients, we examined the gene expression levels of IGF-IR, IR, IGF-I and IRS-1 by using quantitative PCR. IGF-II was never autocrinely produced by EWS tumours (data not shown). We examined 57 primary tumours (Table 2; median follow-up 88 months; range 16–192 months), 7 local recurrences and 7 distant lung or bone metastasis (recurrences and metastasis derived from different patients). All the samples expressed these genes without significant differences

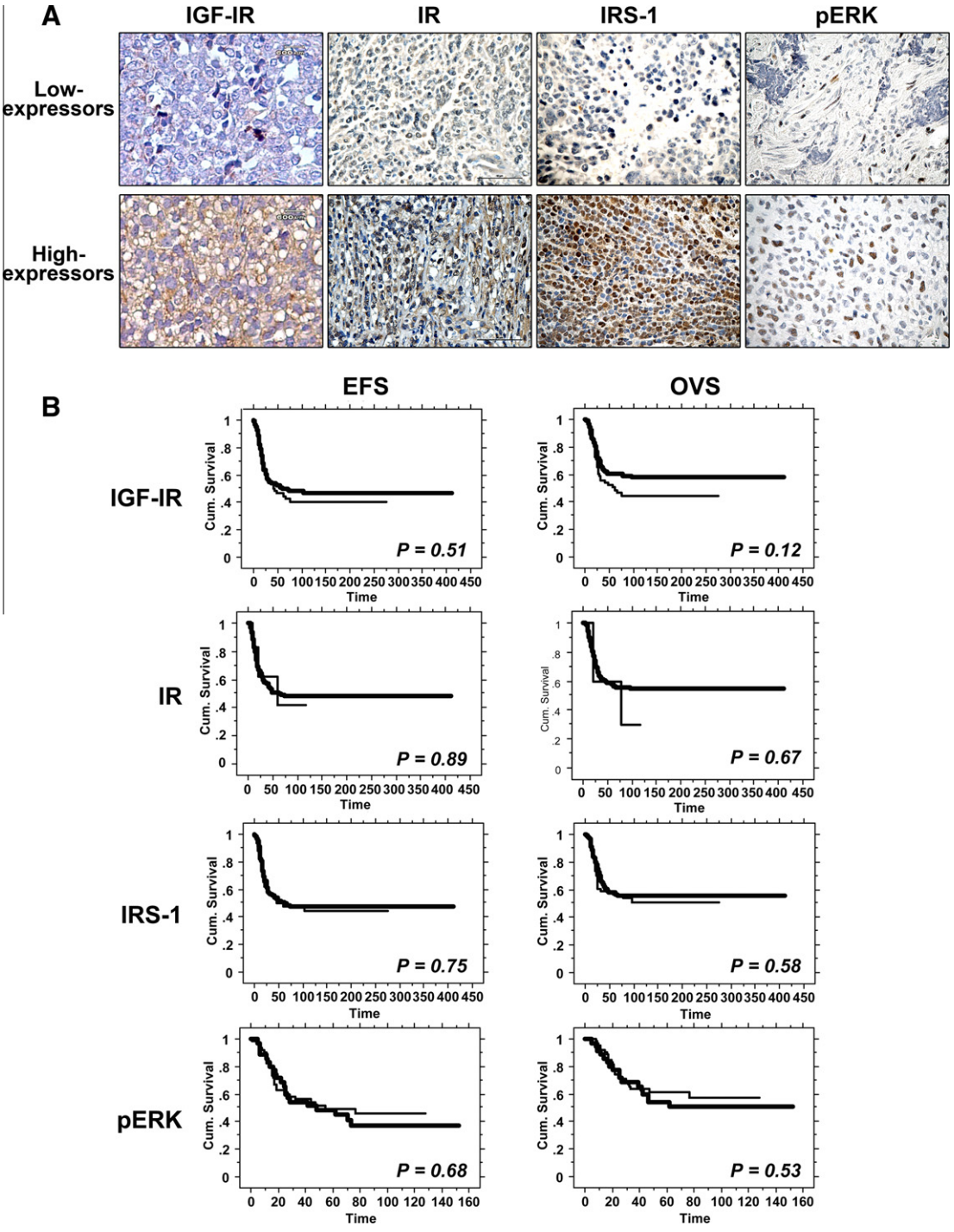

Fig. 1 – Representative expression of IGF-IR, IR, IRS-1 and p-Erk in EWS tissue array samples by immunohistochemistry (A). Regarding IGF-IR, IR and IRS-1, Cases were classified as 'low-expressors', when no staining or low positivity was observed, and 'high expressors' when medium-high positivity was present. For p-ERK, samples were scored as positive or negative. Survival curves were designed accordingly. Comparison of survival curves was performed by the log-rank test. Time scale refers to months from diagnosis (B). Thick lines indicate IGF-IR, IR or IRS-1 high expressing and pERK-positive patients. EFS, event-free survival; OVS, overall survival.

among primary tumours, recurrences and metastases (data not shown). In primary tumours, we evaluated whether gene expression level was associated with the clinical outcome of patients. For each gene, the median expression value was calculated, and patients were stratified as 'high-expressors' or 'low-expressors' relative to the median value (Supplementary Table 1). IGF-I, IGF-IR and IR showed a statistically significant association with survival (Fig. 2). High levels of expression of the IGF-IR and of its ligand correlated with favourable outcome. The two genes also showed a statistically significant

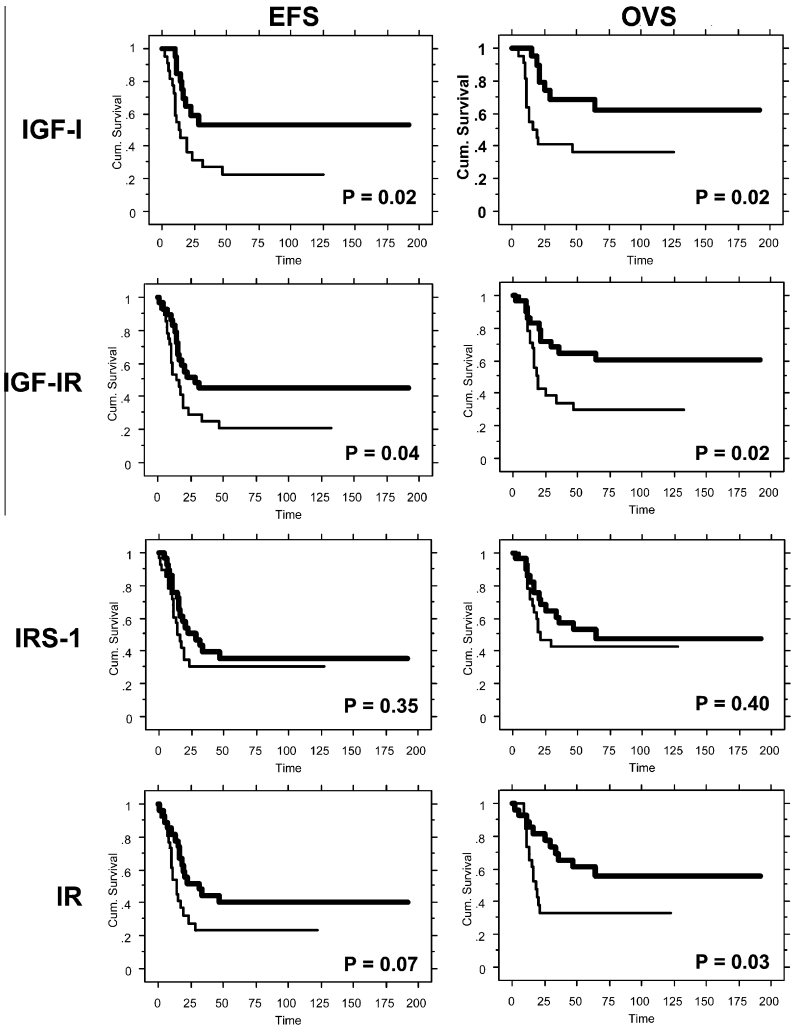

Fig. 2 – Prognostic value of IGF-IR, IGF-I, IRS-1 and IR transcripts on survival of EWS patients with localised tumours at diagnosis. Samples were classified as 'high-expressors' (H) or 'low-expressors' (L), according to median values (Supplementary Table 1). Comparison of survival curves was performed by the log-rank test. Time scale refers to months from diagnosis. Thick lines indicate high expressing patients. EFS, event-free survival; OVS, overall survival.

direct association between them (Pearson correlation test, $r = 0.64$; $p < 0.001$) and with IRS-1 (Pearson correlation test, $r = 0.58$, $p < 0.001$; $r = 0.64$, $p < 0.001$, respectively), confirming that the expression of these major signalling mediators can be used as a surrogate for higher levels of signalling. The expression of IGF-I, IGF-IR and IRS-1 was significantly correlated with the mRNA expression of cyclin D1, a regulator of cell cycle (Pearson correlation test, $r = 0.45$, $r = 0.69$, $r = 0.83$, $p < 0.001$, respectively). Association between IGF-I and cyclin D1 at mRNA level may be explained by the fact that both the genes are targets of the transcriptional factor EWS-FLI1, the aberrant product of the *t*(11;22) chromosomal translocation that is the genetic hallmark of EWS(4). Consistently, also the expression of EWS-FLI1 type 1 and of cyclin D1 (Supplementary Table 1) correlated with favourable clinical outcome ($p = 0.002$ and $p = 0.01$, respectively, data not shown).

The expression of IR did not correlate with any of these genes (IGF-IR, IRS1, cyclin D1 and EWS-FLI1). However, its overexpression also appeared to be associated with better prognosis (Fig. 2). Multivariate analysis considering the five significant biomarkers (IGF-I, IR, IGF-IR, cyclin D1 and EWS-FLI1 type1) confirmed association with prognosis only for IGF-I, and EWS-FLI1 type (Table 3).

3.3. *Circulating levels of IGF-I and IGFBP-3 and relationship with prognosis*

ELISA assay was used to measure circulating levels of IGF-I and IGFBP-3 in the sera of 67 EWS primary patients (median value of 300.6 ng/ml for IGF-I and 2185.4 ng/ml for IGFBP-3) (Supplementary Table 2). Direct correlation between the two soluble proteins was found (Pearson correlation test, $r = 0.63$; $p < 0.001$). When levels of IGF-I and IGFBP-3 of patients with primary tumours was compared with those of 5 patients presenting only metastases (Supplementary Table 2), we observed similar values for IGFBP-3 (median, 2021 ng/ml) but lower levels for IGF-I (102 ng/ml, $p < 0.05$, Mann-Whitney non-parametric test). This may reflect the autocrine production of IGF-I from EWS tumours, which influences the circulating IGF-I levels according to the tumour mass. Patients

Table 3 – Cox proportional hazards regression analysis in 57 EWS patients adjusted for the 5 genes that were significantly associated with prognosis by univariate analysis.

Variable	Adjusted risk rate ratio	95% Confidence interval	*p* Value
IGF-I	2.41	1.03–5.62	0.04
IGF-IR	1.45	0.56–3.82	0.44
IR	1.82	0.75–4.50	0.18
Cyclin D1	1.12	0.41–3.07	0.89
EWS/FLI1	3.44	1.28–9.26	0.01

were stratified as 'high expressors' or 'low expressors' according to the median value (Supplementary Table 2). Clinicopathologic features of these patients are reported in Table 2 (median follow-up 50 months; range 16–180 months). Among clinical parameters, a significant association with survival was found for percentage of necrosis after preoperative chemotherapy and for surgery (Table 2). Regarding biomarkers here considered, high expressors of IGF-I showed better prognosis (EFS, $p = 0.06$; OVS, $p = 0.02$) (Fig. 3). IGFBP-3 is known to link IGF-I and is required for its circulation in the blood. Thus, a similar trend was observed for the expression of this protein, high levels being associated with better survival (Fig. 3). Since at local level, IGFBP-3 is known to inhibit and sequester IGF-I, the ratios of IGFBP-3 to IGF-I was also considered. No significant correlation with clinical outcome was observed.

4. Discussion

Understanding the clinical role for IGF system may be very important in the treatment of Ewing's sarcoma patients because different subtypes of patients have distinct outcome and may require different treatment protocol. We have demonstrated high tumour expression of IGF-IR and IR in around 70-80% of patients. In most of the cases both the receptors are present in the same tumour. However, in the few (less that 10%) cases that are negative for IGF-IR, IR was present and vice versa. This may be important to explain why the recent clinical studies with truly selective anti-IGF-IR human antibodies had demonstrated excellent results only in a very minority of patients (less than 10%).[11] The role of IR in eliciting resistance to anti-IGF-IR therapies has been recently highlighted.[17,18] Cells resistant to the anti-IGF-IR therapies overexpressed IR and may be sustained in their growth by autocrine loops mediated by IGFs and/or insulin. IGF-1 is constantly produced by EWS cells and it can also bind IR, although with lower affinity than IGF-IR.[16] Considering this recent information and the expression of both the receptors in the same tumour, it is conceivable that only the few EWS tumours that express IGF-IR but not IR (15/290, 5% of the

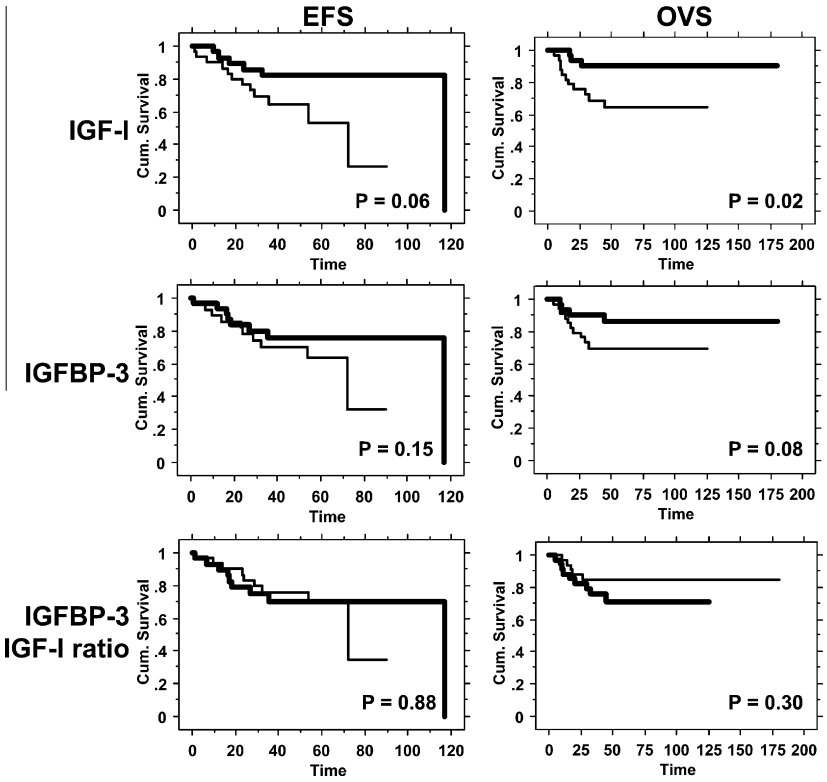

Fig. 3 – Prognostic value of circulating levels of IGF-I on survival of EWS patients with localised tumours at diagnosis. Samples were classified as 'high-expressors' (H) or 'low-expressors' (L), according to median values (Supplementary Table 2). Comparison of survival curves was performed by the log-rank test. Time scale refers to months from diagnosis. Thick lines indicate patients with high IGF-I or IGFPBP3 serum levels or high IGFBP3/IGF-I ratios. EFS, event-free survival; OVS, overall survival.

cases) dramatically benefit from therapies specifically targeting IGF-IR.

With respect to conventional treatments, high expression of IGF-IR or IR by immunohistochemistry did not seem to distinct patients with different prognosis. However, immunohistochemistry has limited power in terms of antigen quantification. Thus, for a smaller series (57 patients) we analysed also the mRNA expression level of IGF-IR, IR, IGF-I and IRS-1 by using quantitative PCR. Our findings support a relationship between high expression of IGF-IR, IR and IGF-I and favourable prognosis. This association was also confirmed for serum levels of IGF-I: high circulating levels of IGF-I appeared as a favourable indicator of outcome. These findings are in line with two previous studies in sarcoma and some sporadic evidences in carcinomas. In EWS, Toretzky et al.[20] showed that lower IGF-I circulating levels were found in patients with metastatic disease. In soft tissue sarcomas, a significant association was shown between high expression of IGF-IR and favourable outcome.[21] Immunohistochemistry of primary breast tumours and matched control samples revealed that IGF-IR and IRS-1 were expressed at high levels in control tissues and in well or moderately differentiated carcinoma, but at low levels in poorly differentiated cancers.[22] In addition, high mRNA expression of IGF-I was associated with lower risk of disease recurrences in breast patients.[23] Similarly, IGF-IR levels were significantly reduced in prostate carcinoma, as compared with benign prostate epithelium,[24] thus supporting the view of IGF-IR as a marker of differentiation. Similar association with outcome has been reported for IR by Mathieu et al.[25] and by Mulligan et al.[26] in breast cancer. Overall, although conflicting data exist in the literature, these studies, including ours, are in contrast with the common view of IGF-IR as a marker of aggressiveness and indicate that in some cancers the transition to frank malignancy and poor treatment responsiveness seem to be associated with a reduction of IGF system activity. Explanation for these findings is not immediate and further research to enhance our understanding of the potential cross reactivity of insulin and IGFs with their respective receptors and hybrid receptors as well as biological effects of IGF system is recommended. IGF system may indeed sustain tumour cell proliferation, protect cells from apoptosis and DNA damage and favour differentiation.[12] Although many of these aspects have been molecularly dissected, there are still issues of the IGF-IR system biology that remains obscure, such as its role in cell differentiation. Association with chemotherapeutic effects is also another level of complexity. Although some previous experimental evidences, including ours, have shown that treatment of breast cancer and EWS cells with anti-IGF-IR antibodies can sensitise these cells to doxorubicin *in vitro*,[27,28] other studies have demonstrated that increased IGF-I signalling is correlated with higher sensitivity to various chemotherapeutic agents[29] suggesting that our understanding of this relationship still need improvements. It is likely that cells respond to autocrine and paracrine IGFs/insulin to maintain an appropriate balance between proliferation and differentiation.[30] Disruption of this balance (i.e. excess of IGF secretion, IGF-IR overexpression) may lead to increased cellular proliferation, that in turn makes cells more sensitive to truly anti-proliferative agents, such as doxorubicin or vincristine. Acting as a growth regulator and sustaining the proliferation rate of the cells, the receptors may maintain the higher chemosensitivity of high-expressing tumours. On the other hand, high expression of IGF-I and receptors may also indicate higher levels of cell differentiation, as suggested in breast cancer. IGF system is indeed implicated in promoting the differentiation of mesenchymal stem cells,[15] the putative cells of origin of EWS.[31] In addition, our results seem to indicate a different role for IR and IGF-IR. In fact, while IGF-IR expression was found to be associated with the expression of the ligand IGF-I, the main intracellular mediators IRS1 and p-ERK as well as with proliferating markers, such as cyclin D1 expression, this was not the case for IR. IR expression seems to be independently associated with patient prognosis. Verifying whether and which biological effects of IR may be relevant for EWS patient outcome is required. In addition, replication of our findings in other datasets, and further exploration of assay methods is recommended to examine optimal cut-points for prognostic effects.

All together, the results obtained in this large series, for a rare tumour, of patients support some novel and clinically important conclusion: 1. IGF-IR and IR are expressed in virtually all EWS tumours; usually both the receptors are present but when one receptor is lacking the other one is always present. 2. Evaluation of IGF-IR, IR and IGF-I with quantitative methods may discriminate differential levels of expression with influences on the patients outcome; 3. The significance of IGF-IR, IR and IGF-I as indicators of patient outcome is not the obvious one and suggests the need of further research before combined treatments with conventional and anti-IGF-IR/IR drugs can be optimally designed.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.01.007.

REFERENCES

1. Bacci G, Forni C, Longhi A, et al. Long-term outcome for patients with non-metastatic Ewing's sarcoma treated with adjuvant and neoadjuvant chemotherapies. 402 patients treated at Rizzoli between 1972 and 1992. *Eur J Cancer* 2004;**40**(1):73–83.
2. Grier HE, Krailo MD, Tarbell NJ, et al. Addition of ifosfamide and etoposide to standard chemotherapy for Ewing's sarcoma and primitive neuroectodermal tumor of bone. *N Engl J Med* 2003;**348**(8):694–701.

3. Subbiah V, Anderson P, Lazar AJ, et al. Ewing's sarcoma: standard and experimental treatment options. *Curr Treat Options Oncol* 2009;**10**(1–2):126–40.
4. Ordonez JL, Osuna D, Herrero D, de Alava E, Madoz-Gurpide J. Advances in Ewing's sarcoma research: where are we now and what lies ahead? *Cancer Res* 2009;**69**(18):7140–50.
5. Barr RD, Wunder JS. Bone and soft tissue sarcomas are often curable–but at what cost?: a call to arms (and legs). *Cancer* 2009;**115**(18):4046–54.
6. Wunder JS, Nielsen TO, Maki RG, O'Sullivan B, Alman BA. Opportunities for improving the therapeutic ratio for patients with sarcoma. *Lancet Oncol* 2007;**8**(6):513–24.
7. Toguchida J, Nakayama T. Molecular genetics of sarcomas: applications to diagnoses and therapy. *Cancer Sci* 2009;**100**(9):1573–80.
8. Lessnick SL, Dei Tos AP, Sorensen PH, et al. Small round cell sarcomas. *Semin Oncol* 2009;**36**(4):338–46.
9. Scotlandi K, Picci P. Targeting insulin-like growth factor 1 receptor in sarcomas. *Curr Opin Oncol* 2008;**20**(4):419–27.
10. Olmos D, Tan DS, Jones RL, Judson IR. Biological rationale and current clinical experience with anti-insulin-like growth factor 1 receptor monoclonal antibodies in treating sarcoma: twenty years from the bench to the bedside. *Cancer J* 2010;**16**(3):183–94.
11. Olmos D, Postel-Vinay S, Molife LR, et al. Safety, pharmacokinetics, and preliminary activity of the anti-IGF-1R antibody figitumumab (CP-751,871) in patients with sarcoma and Ewing's sarcoma: a phase 1 expansion cohort study. *Lancet Oncol* 2010;**11**(2):129–35.
12. Samani AA, Yakar S, LeRoith D, Brodt P. The role of the IGF system in cancer growth and metastasis: overview and recent insights. *Endocr Rev* 2007;**28**(1):20–47.
13. Pollak M. Targeting insulin and insulin-like growth factor signalling in oncology. *Curr Opin Pharmacol* 2008;**8**(4):384–92.
14. Scotlandi K, Benini S, Sarti M, et al. Insulin-like growth factor I receptor-mediated circuit in Ewing's sarcoma/peripheral neuroectodermal tumor: a possible therapeutic target. *Cancer Res* 1996;**56**(20):4570–4.
15. Cironi L, Riggi N, Provero P, et al. IGF1 is a common target gene of Ewing's sarcoma fusion proteins in mesenchymal progenitor cells. *PLoS One* 2008;**3**(7):e2634.
16. Belfiore A, Frasca F, Pandini G, Sciacca L, Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr Rev* 2009;**30**(6):586–623.
17. Ulanet DB, Ludwig DL, Kahn CR, Hanahan D. Insulin receptor functionally enhances multistage tumor progression and conveys intrinsic resistance to IGF-1R targeted therapy. *Proc Natl Acad Sci USA* 2010;**107**(24):10791–8.
18. Buck E, Gokhale PC, Koujak S, et al. Compensatory insulin receptor (IR) activation on inhibition of insulin-like growth factor-1 receptor (IGF-1R): rationale for cotargeting IGF-1R and IR in cancer. *Mol Cancer Ther* 2010;**9**(10):2652–64.
19. Kojima T, Asami S, Chin M, et al. Detection of chimeric genes in Ewing's sarcoma and its clinical applications. *Biol Pharm Bull* 2002;**25**(8):991–4.
20. Toretsky JA, Steinberg SM, Thakar M, et al. Insulin-like growth factor type 1 (IGF-1) and IGF binding protein-3 in patients with Ewing sarcoma family of tumors. *Cancer* 2001;**92**(11):2941–7.
21. Ahlen J, Wejde J, Brosjo O, et al. Insulin-like growth factor type 1 receptor expression correlates to good prognosis in highly malignant soft tissue sarcoma. *Clin Cancer Res* 2005;**11**(1):206–16.
22. Schnarr B, Strunz K, Ohsam J, et al. Down-regulation of insulin-like growth factor-I receptor and insulin receptor substrate-1 expression in advanced human breast cancer. *Int J Cancer* 2000;**89**(6):506–13.
23. Mu L, Katsaros D, Wiley A, et al. Peptide concentrations and mRNA expression of IGF-I, IGF-II and IGFBP-3 in breast cancer and their associations with disease characteristics. *Breast Cancer Res Treat* 2009;**115**(1):151–62.
24. Plymate SS, Bae VL, Maddison L, Quinn LS, Ware JL. Type-1 insulin-like growth factor receptor reexpression in the malignant phenotype of SV40-T-immortalized human prostate epithelial cells enhances apoptosis. *Endocrine* 1997;**7**(1):119–24.
25. Mathieu MC, Clark GM, Allred DC, Goldfine ID, Vigneri R. Insulin receptor expression and clinical outcome in node-negative breast cancer. *Proc Assoc Am Physicians* 1997;**109**(6):565–71.
26. Mulligan AM, O'Malley FP, Ennis M, Fantus IG, Goodwin PJ. Insulin receptor is an independent predictor of a favorable outcome in early stage breast cancer. *Breast Cancer Res Treat* 2007;**106**(1):39–47.
27. Benini S, Manara MC, Baldini N, et al. Inhibition of insulin-like growth factor I receptor increases the antitumor activity of doxorubicin and vincristine against Ewing's sarcoma cells. *Clin Cancer Res* 2001;**7**(6):1790–7.
28. Beech DJ, Parekh N, Pang Y. Insulin-like growth factor-I receptor antagonism results in increased cytotoxicity of breast cancer cells to doxorubicin and taxol. *Oncol Rep* 2001;**8**(2):325–9.
29. Gooch JL, Van Den Berg CL, Yee D. Insulin-like growth factor (IGF)-I rescues breast cancer cells from chemotherapy-induced cell death–proliferative and anti-apoptotic effects. *Breast Cancer Res Treat* 1999;**56**(1):1–10.
30. Baserga R. The contradictions of the insulin-like growth factor 1 receptor. *Oncogene* 2000;**19**(49):5574–81.
31. Tirode F, Laud-Duval K, Prieur A, et al. Mesenchymal stem cell features of Ewing tumors. *Cancer Cell* 2007;**11**(5):421–9.