EUROPEAN JOURNAL OF CANCER 48 (2012) 1570–1580

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p53 Regulates insulin-like growth factor-I receptor gene expression in uterine serous carcinoma and predicts responsiveness to an insulin-like growth factor-I receptor-directed targeted therapy

Zohar Attias-Geva [a], *Itay Bentov* [a], *Dvora Kidron* [b], *Keren Amichay* [c], *Rive Sarfstein* [a], *Ami Fishman* [c], *Ilan Bruchim* [c], *Haim Werner* [a,*]

[a] *Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*
[b] *Division of Pathology, Meir Medical Center, Kfar Sava, Israel*
[c] *Gynecologic Oncology Unit, Department of Obstetrics and Gynecology, Meir Medical Center, Kfar Sava, Israel*

ARTICLE INFO

Article history:
Available online 25 October 2011

Keywords:
Insulin-like growth factor-I (IGF-I)
IGF-I receptor
Endometrial cancer
p53
Transcription regulation

ABSTRACT

The role of the insulin-like growth factors (IGF) in endometrial cancer has been well established. The IGF-I receptor (IGF-IR), which mediates the biological actions of IGF-I, is usually overexpressed in endometrial tumours. Uterine serous carcinoma (USC) constitutes a defined histological category among endometrial cancers. Mutation of the p53 gene appears early in the course of the disease and is considered a key event in the initiation of USC. The aim of the present study was to evaluate the potential interactions between p53 and the IGF-IR in USC. In addition, we investigated the role of p53 as a biomarker in IGF-IR targeted therapies. Immunohistochemical analysis in a collection of 35 USC specimens revealed that IGF-IR is highly expressed in primary and metastatic USC. Likewise, p53 was expressed in 85.7% of primary tumours and 100% of metastases. A significant negative correlation between p53 expression and survival was noticed. In addition, using USC-derived cell lines we provide evidence that p53 regulates IGF-IR gene expression *via* a mechanism that involves repression of the IGF-IR promoter. We show that the mechanism of action of p53 involves interaction with zinc finger protein Sp1, a potent transactivator of the IGF-IR gene. Finally, we demonstrate that USC tumours overexpressing p53 are more likely to benefit from anti-IGF-IR therapies. In summary, we provide evidence that p53 regulates IGF-IR gene expression in USC cells *via* a mechanism that involves repression of the IGF-IR promoter. The interplay between the p53 and IGF-I signalling pathways is of major basic and translational relevance.

1. Introduction

Endometrial cancer is the most widespread gynaecologic cancer in Western countries, with more than 40,000 new cases in 2009 in the United States alone. The incidence of the disease has been increasing in recent years presumably as a result of the growing obesity epidemic.[1] Endometrial cancers are classified into two major categories based on light microscope appearance, clinical behaviour and epidemiology.[2] Type I tumours, being the most frequent, are usually

* *Corresponding author:* Tel.: +972 3 6408542; fax: +972 3 6406087.
E-mail address: hwerner@post.tau.ac.il (H. Werner).
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doi:10.1016/j.ejca.2011.09.014

oestrogen-dependent, low-grade neoplasms and are generally associated with a relatively good prognosis. Type II tumours, on the other hand, are usually diagnosed at an advanced age, are not associated with exposure to oestrogens, display a less differentiated phenotype and have a worst prognosis. Uterine serous carcinoma (USC) constitutes the predominant histological class among Type II tumours.[3] USC is diagnosed at an advanced stage and accounts for 50% of all endometrial cancers relapses, with a 5-year survival rate of 55%. While early-stage endometrial cancers are highly curable (mostly by surgery alone), the prognosis of invasive and metastatic endometrial cancer is poor.[4] Mutations of the p53 gene appear early in the course of the disease[5] and are considered a paramount genetic factor in the initiation of USC.[6] Overexpression of p53 in endometrial carcinoma is usually correlated with advanced disease and poor prognosis.[7] In early (stage I) endometrial cancer, p53 overexpression is a poor prognostic marker[8] and the same behaviour was observed for p53 in USC.[9] Both mutation-dependent and independent p53 inactivations in USC are associated with nuclear overexpression.[10] Of interest, positive staining for p53 in USC is not necessarily indicative of a genetic mutation.[11] The molecular targets of p53 in endometrial cancer, however, are yet to be identified.

The insulin-like growth factors (IGF-I, IGF-II) have a major role in uterine development and physiology and are also involved in endometrial cancer. Cyclic changes in IGF-I expression and action play an important role in regulating the transition of premenopausal endometrium through proliferative, secretory and menstrual cycles.[12,13] The biological actions of the IGFs are mediated by the IGF-I receptor (IGF-IR), a transmembrane tetramer structurally related to the insulin receptor.[14,15] The IGF-IR mediates strong antiapoptotic signals and is usually overexpressed in endometrial tumours.[16–19] In accordance with its important role in both tumour initiation and progression, IGF-IR blockade emerged in recent years as a promising treatment modality.[20] A number of approaches are currently being explored for this purpose in various types of neoplasm, including the use of small molecular weight tyrosine kinase inhibitors as well as IGF-IR blocking monoclonal antibodies.[21–24] In this context, it is of major importance to identify biomarkers that may predict responsiveness to IGF-IR targeted therapies.

The IGF-IR is expressed by virtually every tissue during development, and several hormonal factors responsible for IGF-IR regulation have been identified.[25–27] Furthermore, transcription factors controlling IGF-IR gene expression under both physiological and pathological conditions have been identified (e.g. BRCA1, E2F1, Wilm's tumour-1, etc), and the mechanisms of action of some of these nuclear proteins have been delineated.[28–30] p53 was shown to suppress IGF-IR promoter activity by ~90% as well as endogenous IGF-IR mRNA levels in osteosarcoma and other cell types. In contrast, mutant forms of p53 enhanced IGF-IR gene expression in cells with a null-p53 background.[31,32] In addition, the activation status of the p53-Mdm2 axis was shown to be regulated by IGF-I, suggesting the existence of a complex loop that regulates in a coordinate fashion the bioactivities of the IGF-I and p53 signalling pathways. No studies have so far addressed the potential interactions between p53 and the IGF-IR in endometrial cancer.

In view of the important roles of p53 and the IGF-IR in endometrial cancer, and to expand our previous studies on the interactions between p53 and the IGF system, we evaluated in the present study (i) the expression of p53 and the IGF-IR in a collection of USC specimens; (ii) the correlation between p53 and IGF-IR expression and survival; (iii) the regulation of IGF-IR gene expression by p53; and (iv) the potential role of p53 and IGF-IR as predictors of responsiveness to IGF-IR targeted therapies. Results obtained indicate that IGF-IR is highly expressed in both primary and metastatic USC. A high expression of p53 was also observed in primary and metastatic USC tumours and, furthermore, there was a significant negative correlation between p53 protein expression and survival. Wild type, but not mutant, p53 was able to repress IGF-IR promoter activity in USC-derived cell lines, suggesting that IGF-IR levels are strongly dependent on p53 status. Finally, a blocking anti-IGF-IR monoclonal antibody was capable of decreasing proliferation of a p53-overexpressing, but not a p53-null, USC-derived cell line. Hence, interactions between the p53 and IGF-I signalling pathways seem to have a major role in USC aetiology, and may have broad implications in targeted therapy approaches.

2. Patients and methods

2.1. Patients

A retrospective analysis of all consecutive Jewish patients who were diagnosed with USC in the Gynecologic-Oncology Unit at Meir Medical Center, Kfar Saba, Israel, between 1st April 1997 and 31st December 2007 was performed. Pathologic re-evaluation was performed in all cases, and the diagnosis of USC was based on accepted criteria according to WHO classification.[33] Cases were excluded if the papillary serous component accounted for less than 25% of the surgical specimen, if the predominant histological component was clear cell or if a primary site of origin could not be definitively determined. Clinical characteristics, including age at diagnosis, personal and family history of cancer, ethnic origin (based on a history of three generations) and stage of disease at diagnosis, were abstracted from the patients' medical records. Information regarding the treatment after diagnosis, recurrences and last day of follow-up were retrieved from medical records. Information on patients' vital status was updated through the Israel Population Registry. The study was approved by the Institutional Review Board. The IRB waived informed consent for use of tissue samples from the deceased patients. Written informed consent was obtained from the patients for collection of blood samples and clinical data, following genetic counselling. For deceased patients, formalin-fixed, paraffin-embedded tissue was collected from the Pathology Department, Meir Medical Center.

2.2. Tissue samples and immunohistochemistry (IHC)

All available histological sections of tumours and metastases were reviewed. For each tumour, slides containing adjacent non-neoplastic endometrium (for internal controls), as well as invasion and vascular invasion, were chosen. Archival paraffin blocks were retrieved and 4 μm cuts were made and

placed on SuperFrost[R] Plus glass slides. The following primary antibodies were used: IGF-IRβ [(C-20), SC-713, Santa Cruz Biotechnology (Santa Cruz, CA, USA) dilution 1:100] and p53 [(BP53.12), Zymed Laboratories, dilution 1:50]. Immunohistochemical stainings were carried out using a Ventana Benchmark XT automatic immunostainer.

2.3. Evaluation of immunohistochemical stainings

A 4-degree scale was devised, integrating intensity and extent. Intensity of staining was graded into four categories, as follows: 0 – no staining, 1 – weak staining, 2 – moderate staining and 3 – strong staining. The extensiveness of staining (with no reference to intensity) was graded into two categories: 1 – <50%, 2 – >50%. The *score of staining* was graded according to both intensity and extensiveness, following this scheme:

I No staining = Negative
II Weak up to moderate staining in <50% of tumour cells = Weak/mild
III Weak up to moderate staining in >50% of tumour cells = Moderate/medium
IV Moderate up to strong staining in >50% of tumour cells = Strong

All stains were graded separately by two expert pathologists. Differences in more than 1 grade were present in 5% of cases. Differences derived from fading out and inconstant staining due to variable fixation in regions containing large bulks of tumour. Final scores were agreed upon by both examiners. IGF-IR staining was cytoplasmic. Adjacent endometrium was constantly positive grade 3 and was used as internal control. Staining of p53 was nuclear.

2.4. Survival criteria

Overall survival was measured from the date of primary surgery at diagnosis to the date of death, or for living patients, to the date of last contact. Five-year survival rates were reported as the percent of patients surviving five years after primary surgery. The duration of progression-free interval (PFI) was the interval until progression, death or the date of last contact, whichever came first. Survival curves were constructed according to the Kaplan–Meier method and statistical differences between the curves were calculated with the log-rank test. Multivariate survival analysis was performed using the Cox Proportional Hazards model. A *p*-value of 0.05 was considered statistically significant. SPSS ver.13 program was used for statistical analysis.

2.5. Cell lines and treatments

The human uterine serous carcinoma (USC) cell lines USPC-1 and USPC-2 were employed in this study. Cell lines were derived from USC patients who experienced rapid tumour progression during adjuvant chemotherapy after primary surgical debulking.[34] Cell lines were kindly provided by Dr. A.D. Santin, Yale University School of Medicine, New Haven, CT, USA. USC cells were maintained in RPMI-1640 medium (Biological Industries, Kibbutz Beit Haemek, Israel). Media were supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, and 50 μg/ml gentamicin sulphate. All reagents were purchased from Biological Industries. In addition, 5.6 mg/l fungizone was added (Sigma–Aldrich, St. Louis, MO, USA). Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C. Cells were split twice a week, using Trypsin-Versane (Bio-Lab Laboratories Ltd, Israel) according to their individual confluence and duplication time. In some of the experiments, cells were treated with IGF-I (10 ng/ml) (Cytolab Ltd., Rehovot, Israel), in the absence or presence of cixutumumab (10 μM). Cixutumumab (IMC-A12) (ImClone Systems, New York) is a fully human antibody antagonist to the human IGF-IR. The generation and characterisation of this antibody has been described.[35] Some experiments included Mithramycin A (Sigma–Aldrich), an Sp1-family inhibitor. Mithramycin A was added for 24 h at concentrations of 200 and 500 nM.

2.6. Mutational analysis of p53 in USC cell lines

Mutational analysis of the p53 gene in the USPC-2 cell line revealed a homozygote C to T nucleotide exchange (exon 5: position c.493) which results in the formation of a stop codon at position p.165. The USPC-1 cell line included two polymorphic changes in the p53 gene sequence (intron 3: c.96+41del16bp; exon 4: c.97–29C > A. Mutational analyses were conducted at Galil Genetic Analysis, Kazerin, Israel.

2.7. RT-PCR for IGF-IR mRNA expression

Total RNA was prepared from endometrial cancer cell lines using the Trizol reagent (Sigma–Aldrich). 2.5 μg of total RNA was reverse transcribed and amplified by PCR. The primers used for IGF-IR mRNA were: sense, 5′-TGGAGTGCTGTATGCCTCTG-3′; antisense, 5′-TGATGACCAGTGTTGGCTGG-3′. The size of the band was 275 bp. For control purposes, levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were measured using the following primers: sense, 5′-ACCACAGTCCATGCCATCAC-3′; antisense, 5′- TCCACCACC CTGTTGCTGTA-3′. The size of the amplified GAPDH mRNA band was 452 bp.

2.8. Plasmids and DNA transfections

USPC-1 and USPC-2 cells were seeded in six-well plates the day before transfection and transfected using the JetPEI[TM] reagent (Polyplus Transfection, Illkirch, France), according to manufacturer's recommendations. For transient co-transfection experiments, an IGF-IR promoter luciferase reporter construct, [p(–476/+640)LUC] (nucleotide 1 corresponds to the transcription start site of the rat IGF-IR gene), was employed. Expression vectors encoding wt and mutant (Arg → Trp mutation at position 248) p53 were kindly provided by Dr. Edward Mercer (Thomas Jefferson University, Philadelphia, PA, USA). An Sp1 expression vector including an Sp1 cDNA downstream of an actin promoter (pPact) was provided by Dr. Robert Tjian (University of California, Berkeley, CA, USA). Cells were harvested 48 h after transfection, and luciferase and β-galactosidase (β-gal)

activities were measured. Promoter activities were expressed as luciferase values normalised for β-gal.

2.9. Western immunoblots

Transfected and untransfected cells were harvested with ice-cold phosphate-buffered saline (PBS) containing 5 mmol/L EDTA and lysed in a buffer containing 150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.5), 1% Triton X-100, 2 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L phenylmethylsulphonyl fluoride, 2 μg/mL aprotinin, 1 mmol/L leupeptin, 1 mmol/L pyrophosphate, 1 mmol/L vanadate and 1 mmol/L DTT. Protein content was determined using the Bradford reagent. Samples were electrophoresed through 10% SDS–PAGE, followed by blotting of the proteins onto nitrocellulose membranes. After blocking with 5% skim milk in T-TBS [20 mmol/L Tris-HCl (pH 7.5), 135 mmol/L NaCl, and 0.1% Tween 20], blots were incubated with a rabbit polyclonal anti-human IGF-IR β-subunit antibody (#3027, Cell Signalling Technology, Beverly, MA, USA), washed with T-TBS, and incubated with an horseradish peroxidase-conjugated secondary antibody. In addition, blots were incubated with antibodies against p53 (DO-1 and Pab1801, Santa Cruz Biotechnology), Sp1 (PEP 2, Santa Cruz Biotechnology) and tubulin (B-5-1-2, Sigma–Aldrich). Proteins were detected using the SuperSignal West Pico® Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

2.10. Co-immunoprecipitation analyses

Transfected cells were harvested and lysed as described above. Cellular extracts were immunoprecipitated with antibodies against p53 (DO-1 and Pab1801) and Sp1 (PEP-2). Immunoprecipitates were resolved on 8% SDS–PAGE and immunoblotted with anti-Sp1 or anti-p53 antibodies, washed and incubated with horseradish peroxidase-conjugated secondary antibody. p53 and Sp1 were detected using the SuperSignal West Pico® Chemiluminescent Substrate.

2.11. Proliferation assays

To determine the effect of IGF-IR inhibition on cell growth, endometrial cancer cells were seeded at a density of 2×10^4 cells per well in 24-well plates and allowed to attach for 24 h. Cells were then treated with the inhibitor and cultured for 72 h, in triplicate dishes. Every 24 h, 100 μl of Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma–Aldrich) was added to each well and incubated at 37 °C for 1 h, after which the medium was removed and 300 μl of dimethylsulphoxide (DMSO, Sigma–Aldrich) was added. The colour developed was quantitated by measuring absorbance at a wavelength of 530 nm and reference wavelength of 630 nm on a UVmax Kinetic Microplate Reader (Molecular Devices Inc., Sunnyvale, CA, USA).

2.12. Statistical analysis

The statistical significance of the differences observed between groups was assessed using the Chi-square, *t*-test or Mann-Whitney rank test, each when appropriate. Kaplan–Meier was used for survival analyses. $p < 0.05$ was considered statistically significant. The data were analysed using SPSS-17.

3. Results

3.1. Immunohistochemical analysis of IGF-IR and p53 expression in primary and metastatic endometrial tumours

The study group consisted of 35 Jewish patients diagnosed with USC between 1st April 1997 and 31st December 2007. Seventeen out of the 35 patients had metastatic tumours. The mean age at diagnosis was 71 ± 9.3 years (range 47–87) with a median age of 73 years. Results of immunohistochemical analysis revealed that IGF-IR is highly expressed in USC, both in primary and metastatic tumours (Fig. 1). Positive staining (grades II–IV) was observed in 94.3% of primary USC tumours and 100% of metastases. A high expression of IGF-IR (grades III and IV) was recorded in 42.8% of the primary tumours and 77.8% of the metastatic tumours. p53 was expressed in 85.7% of primary tumours and 100% of metastases. The staining was strong (grade IV) in 68.6% of primary USC tumours and 100% of metastases (Fig. 2). We found no correlation between IGF-IR or p53 protein expression to age at diagnosis, previous hormonal therapy, stage of disease, family history and BRCA1/2 mutation carrier status. Moreover, we found no correlation between the IGF-IR and p53 protein expression in these tumours.

3.2. Survival of women with USC based on p53 status

We observed a significant negative correlation between p53 protein expression and survival. Five-years survival was 12% in patients with strong p53 staining compared to 60% in patients with negative-moderate expression. Mean PFI was significantly shorter in patients with strong p53 expression compared to patients with negative-moderate p53 expression (28 versus 58.2 months, respectively; $p = 0.012$) in univariate analysis.

Kaplan–Meier survival analysis was performed for patients whose tumours exhibited overexpression of p53 and for patients whose tumours did not express p53 (Fig. 3). The Mantel–Cox log rank test was used to compare the survival curves. There was a significantly shorter survival in patients whose tumours overexpressed p53, compared to those whose tumours did not express p53 ($p = 0.009$). No correlation between IGF-IR expression and survival was observed.

3.3. IGF-IR and p53 expression in USC-derived cell lines

To begin to investigate the potential involvement of tumour suppressor p53 in transcriptional regulation of the IGF-IR gene, we measured in initial experiments the basal IGF-IR levels in USC-derived cell lines. Western blot analysis revealed that USPC-1 cells express high levels of IGF-IR whereas USPC-2 cells express reduced IGF-IR levels (Fig. 4A). To assess whether the IGF-IR levels were associated with corresponding trends in mRNA expression, IGF-IR mRNA levels were measured using a semiquantitative RT-PCR assay. Results obtained showed that

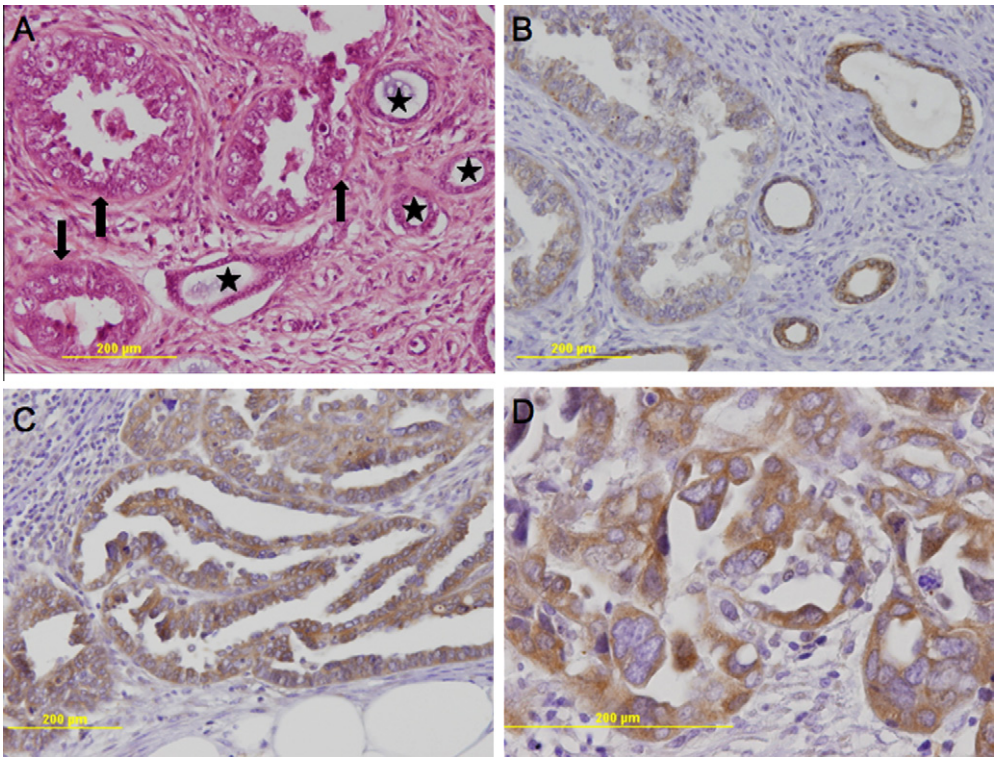

Fig. 1 – Immunohistochemical staining of IGF-IR in primary and metastatic USC. (A) Primary tumour, neoplastic glands (arrows) adjacent to non-neoplastic endometrial glands (asterisks). H&E, ×200. (B) *Primary tumor*, weak cytoplasmatic staining in <50% of cells (grade II) in neoplastic glands. Moderate staining in >50% of cells in adjacent non-neoplastic glands. (C) *Metastasis*, moderate cytoplasmatic staining in nearly all neoplastic cells. (D) *Metastasis*, a focus of increased atypia. Moderate and strong staining in all neoplastic cells.

IGF-IR mRNA levels correlated with protein levels (Fig. 4B). In addition, Western blots revealed that USPC-1 cells express high levels of p53 whereas USPC-2 cells express very low p53 levels (Fig. 4C).

3.4. *Regulation of IGF-IR gene expression by p53*

We have shown in previous studies that IGF-IR gene expression is modulated by tumour suppressor p53 in osteosarcoma-derived Saos-2 cells, lacking endogenous p53.[31] Specifically, whereas wild-type p53 suppressed IGF-IR promoter activity, mutant versions of p53 significantly enhanced promoter activity. To examine the potential involvement of p53 in regulation of IGF-IR gene expression in USC, cells were transiently co-transfected with a p53 expression vector along with an IGF-IR promoter-luciferase reporter plasmid [p(–476/+640)LUC]. Results of luciferase measurements indicate that expression of wild-type p53 in USPC-2 cells (expressing an endogenous truncated p53) repressed IGF-IR promoter activity by ~65%. Mutant p53, however, was unable to inhibit promoter activity (Fig. 5A). On the other hand, in USPC-1 cells, expression of wild-type p53 had essentially no effect on IGF-IR promoter activity, most probably due to the fact that USPC-1 cells express high concentrations of endogenous p53 that obscure the effects of the transfected protein (Fig. 5B). The increase in p53 level in USPC-1 cells following transfection is shown in Fig. 5C.

3.5. *Functional interactions between p53 and Sp1 in transcriptional regulation of the IGF-IR gene*

Previous studies have identified a number of potential binding sites for transcription factor Sp1 in the proximal region of the IGF-IR promoter.[36,37] Furthermore, Sp1 was shown to strongly transactivate the IGF-IR promoter and to interact with a number of tumour suppressors, including p53.[32] To investigate the involvement of Sp1 in regulation of IGF-IR gene expression by p53, Sp1 levels were measured in USPC-1 and USPC-2 cells. As shown in Fig. 6A, the pattern of Sp1 expression in USC cell lines was diametrically opposed to that of p53. Specifically, USPC-1 cells express low Sp1 levels whereas USPC-2 cells contain high Sp1 concentrations. To address the functional interactions between p53 and Sp1 in regulation of the IGF-IR gene, transient co-transfections were performed in USPC-2 cells using p53 and Sp1 expression vectors, along with the p(–476/+640)LUC reporter. In these cells, Sp1 induced a ~25% stimulation of the IGF-IR promoter, while p53 suppressed ~60% of the Sp1-induced transactivation (Fig. 6B). Sp1, however, had no effect on IGF-IR promoter activity in USPC-1 cells (Fig. 6C).

3.6. *Inhibition of IGF-IR gene expression by Mithramycin A*

To more directly examine the involvement of Sp1 on IGF-IR gene expression, the Mithramycin A inhibitor was employed. Mithramycin A has been shown to block the activity of

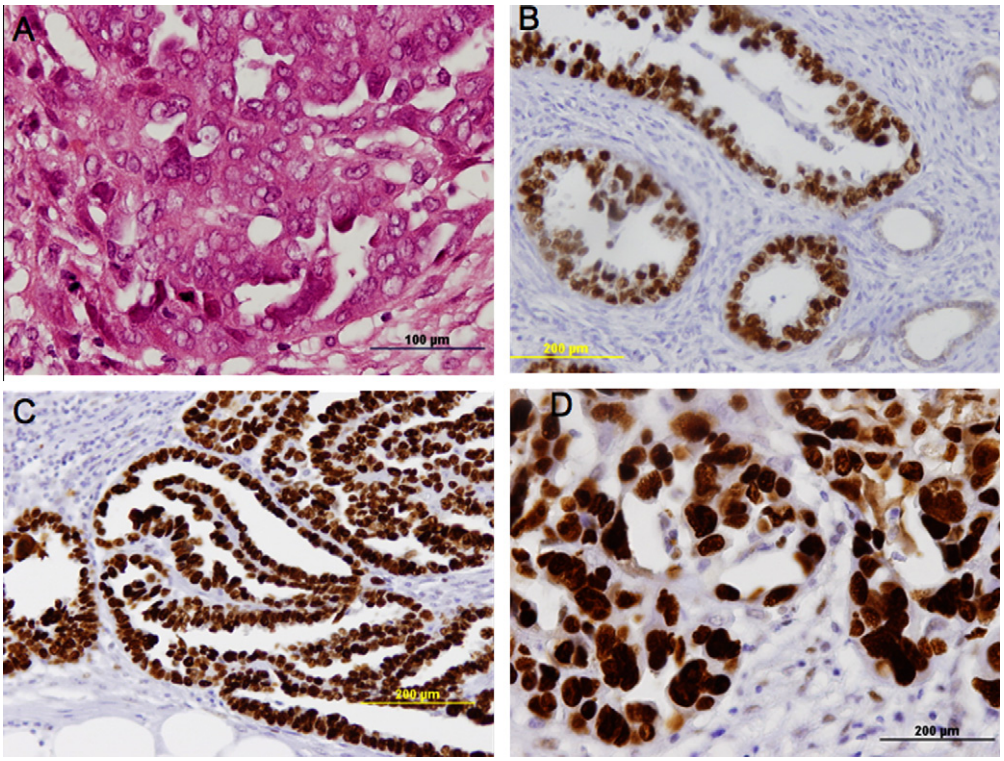

Fig. 2 – Immunohistochemical staining of p53 in primary and metastatic USC. (A) A focus of increased atypia in metastatic tumour. Enlarged, pleomorphic and bizarre nuclei, atypical mitoses. H&E, ×400. (B) *Primary tumor*, strong nuclear staining in nearly all neoplastic cells (grade IV). No staining in adjacent non-neoplastic glands. (C) *Metastasis*, strong nuclear staining in nearly all neoplastic cells (grade IV). (D) *Metastasis*, a focus of increased atypia. Very strong staining in all neoplastic cells (grade IV).

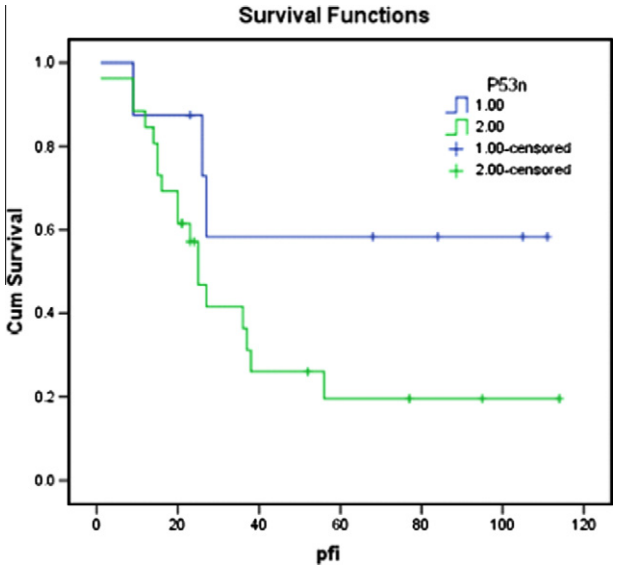

Fig. 3 – Kaplan–Meier survival curve for p53-overexpressing and non-expressing tumours in USC women. PFI, progression free interval; CUM, cumulative survival.

Sp1-family members by binding GC-rich sequences in target promoters. Addition of Mithramycin A (200–500 nM) led to a decrease in IGF-IR promoter activity (Fig. 7A) and

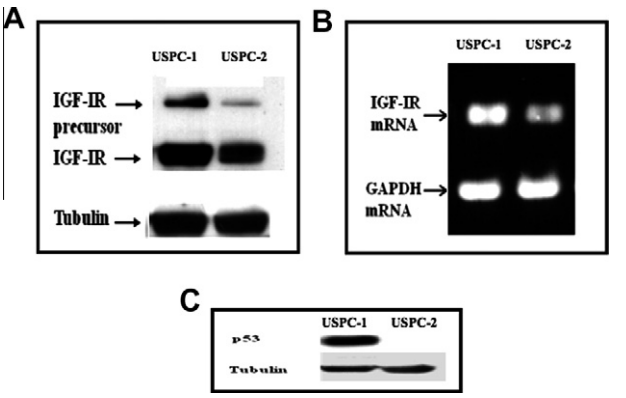

Fig. 4 – Expression of endogenous IGF-IR and p53 in USC-derived cell lines. (A) Western blot analysis of IGF-IR levels in USPC-1 and USPC-2 cell lines. Cells were lysed and extracts were electrophoresed through SDS–PAGE, followed by transfer and incubation with an anti-IGF-IR β-subunit. (B) Total RNA was prepared from endometrial cancer cells and IGF-IR and GAPDH mRNA levels were evaluated by a semi-quantitative RT-PCR assay. (C) Western blot analysis of p53 expression in USPC-1 and USPC-2 cells. Cells were lysed and equal amounts of protein (80 μg) were separated by 10% SDS–PAGE, transferred to nitrocellulose filters, and blotted with anti-p53 (upper panel) or anti-tubulin (lower panel) antibodies. The figure shows a typical blot repeated three times with similar results.

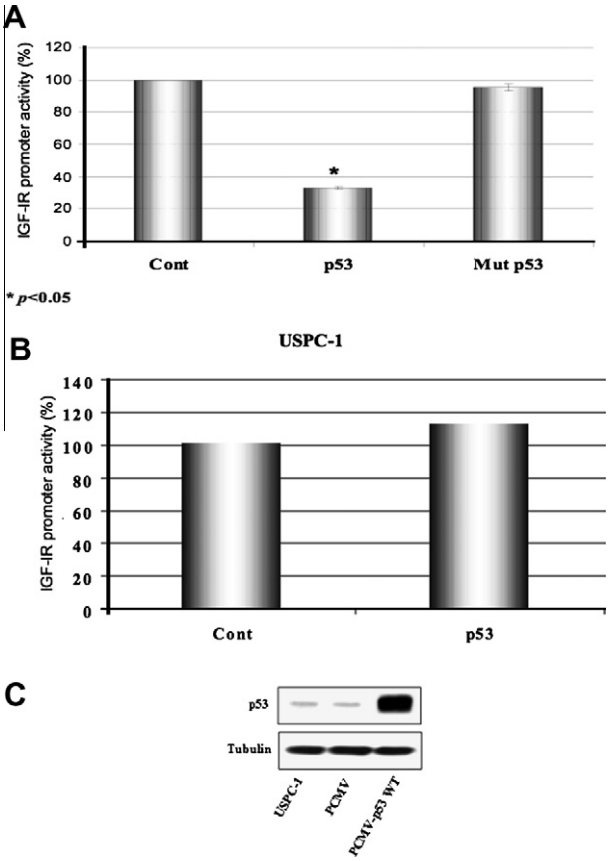

Fig. 5 – Regulation of IGF-IR promoter activity by p53 in USC cells. (A) USPC-2 cells were co-transfected with 1 μg of the p(–476/+640)LUC IGF-IR promoter-luciferase reporter construct, along with 1 μg of the wild-type p53, or codon 248-mutant p53 (or empty vector) and 0.3 μg of the pCMVβ plasmid, using the Jet-PEI™ reagent. Forty hours after transfection cells were harvested and the levels of luciferase and β-galactosidase were measured. Promoter activities are expressed as luciferase values normalised for β-galactosidase. Results are mean ± S.E.M. of three independent experiments, performed in duplicate dishes. *$p < 0.05$ versus control. (B) USPC-1 cells were co-transfected with 1 μg of the p(–476/+640)LUC IGF-IR promoter-luciferase reporter, along with 1 μg of the wild-type P53 expression vector and 0.3 μg of the pCMVβ plasmid, using the Jet-PEI™ reagent. Cells were processed as described above. (C) Western blot of p53 in untransfected cells (USPC-1), cells transfected with empty vector (PCMV), and cells transfected with wild-type p53 (pCMV-p53 WT).

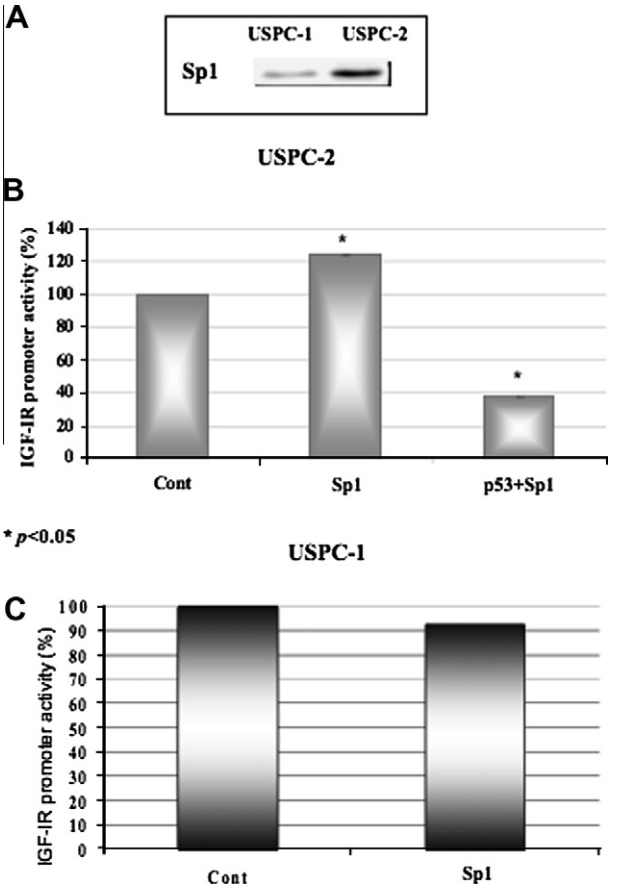

Fig. 6 – Functional interaction between Sp1 and p53 in regulation of IGF-IR promoter. (A) Western blot analysis of Sp1 expression in USPC-1 and USPC-2 cells. (B) USPC-2 cells were co-transfected with 1 μg of the p(–476/+640)LUC IGF-IR promoter-luciferase reporter construct, along with 1 μg of an Sp1 expression vector (pPacSp1, or empty pPac0) in the absence or presence of 1 μg of wild-type p53, and 0.3 μg of the pCMVβ plasmid, using Jet-PEI™. Forty hours after transfection cells were harvested and the levels of luciferase and β-galactosidase were measured. Promoter activities are expressed as luciferase values normalised for β-galactosidase. Results are mean ± S.E.M. of three independent experiments, performed in duplicate dishes. *$p < 0.05$ versus control. (C) USPC-1 cells were co-transfected with 1 μg of the p(–476/+640)LUC IGF-IR promoter-luciferase reporter construct, along with 1 μg of Sp1 expression vector and 0.3 μg of the pCMVβ plasmid. Transfected cells were processed as described above.

endogenous IGF-IR protein levels (Fig. 7B). The inhibitor was especially potent in reducing IGF-IR precursor levels, suggesting that the effect is mainly at the level of biosynthesis of the receptor.

3.7. *Physical interactions between p53 and Sp1*

To investigate whether the functional interactions between p53 and Sp1 were correlated with physical interactions between both proteins, co-immunoprecipitation experiments were performed in USPC-2 cells. For this purpose, cells were transfected with a p53 expression vector, lysed and immunoprecipitated with anti-Sp1 or anti-p53. Precipitates were electrophoresed through 8% SDS–PAGE, transferred to nitrocellulose membranes, and blotted with anti-Sp1 (Fig. 8, upper panel) or anti-p53 (Fig. 8, lower panel). Results obtained show that immunoblotting with anti-p53 identified the 50-kDa protein in anti-Sp1 immunoprecipitates of p53-transfected cells. Likewise, immunoblotting with anti-Sp1 identified the 95-kDa band in anti-p53 precipitates.

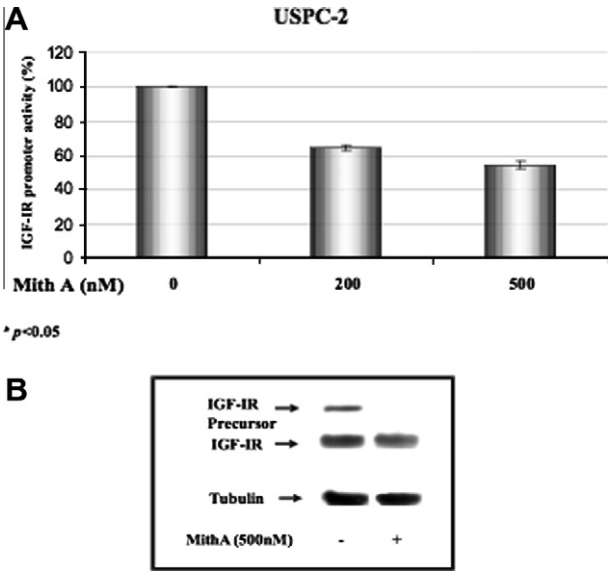

Fig. 7 – Inhibition of IGF-IR gene expression by Mithramycin A. (A) USPC-2 cells were transfected with the p(–476/+640)LUC IGF-IR promoter-luciferase reporter construct. After 24 h, Mithramycin A (200 and 500 nM) was added for 24 h. Control cells were left untreated. Luciferase values were measured as described above. A value of 100% was given to untreated cells. Results are mean ± S.E.M. of 3–4 independent experiments. *$p < 0.05$ versus control cells. (B) USPC-2 cells were incubated with 500 nM Mithramycin A. After 24 h, cells were harvested and IGF-IR levels were assessed by Western blotting.

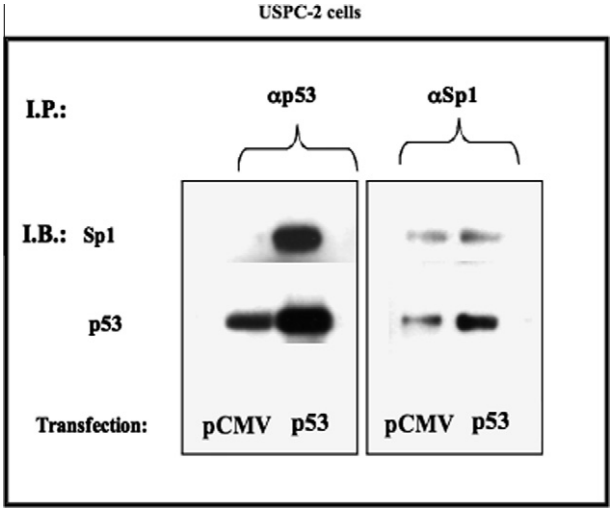

Fig. 8 – Physical interaction between Sp1 and p53. USPC-2 cells were transfected with wild-type p53 (or empty vector). After 48 h cells were lysed and immunoprecipitated with anti-Sp1 or anti-p53. Precipitates were electrophoresed, blotted onto nitrocellulose filters and immunoblotted with anti-p53 or anti-Sp1. I.P., immunoprecipitation; I.B., immunoblotting.

3.8. *Effect of p53 levels on anti-IGF-IR therapies*

Finally, to assess the impact of p53 status on the efficacy of IGF-IR-directed therapies, USPC-1 and USPC-2 cells were seeded in 24-well plates (2×10^4 cells/well), and exposed to cixutumumab, a fully human antibody antagonist to the human IGF-IR for 72 h. The proliferation rate was determined by MTT assays. Results obtained showed that addition of cixutumumab to the USPC-1 cell line (expressing high levels of p53) caused a significant decrease in proliferation rate compared to control cells (Fig. 9, upper panel). On the other hand, the inhibitor had no effect in USPC-2 cells, expressing low levels of p53 (Fig. 9, lower panel).

4. Discussion

The convergence of the p53 and IGF-I signalling pathways has been the focus of considerable basic and translational interest.[38] The present study was designed to evaluate the expression of p53 and the IGF-IR in primary and metastatic USC, to examine the correlation between p53 and IGF-IR expression and survival, to address the regulation of IGF-IR gene expression by p53 and, finally, to define the potential role of p53 and/or IGF-IR as predictors of responsiveness to IGF-IR targeted therapies. Results of immunohistochemical analysis revealed

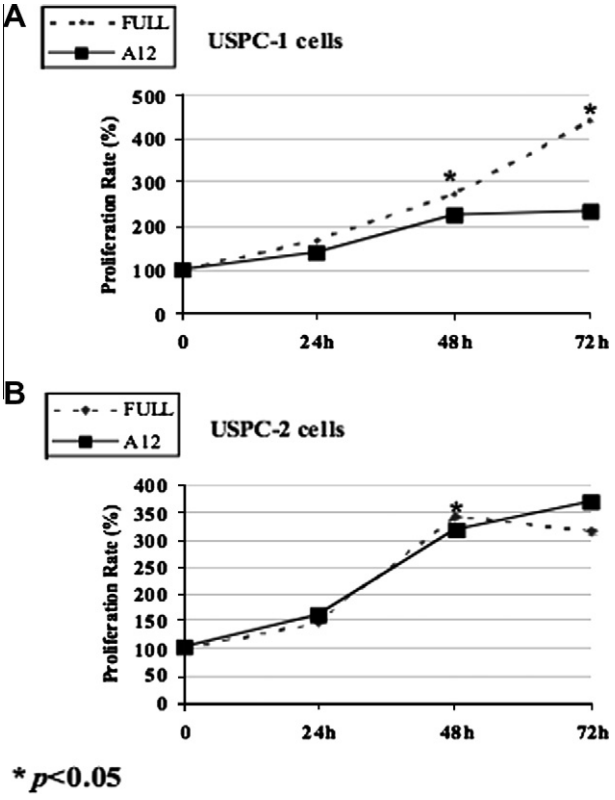

Fig. 9 – Effect of cituxumumab on USPC-1 and USPC-2 cell proliferation. Cells were plated in 24-well plates at a density of 2×10^4 cells/well. The number of cells at time 0 was assigned a value of 100%. The bars represent the mean ± S.E.M. of four independent experiments. *$p < 0.05$ versus time 0.

that IGF-IR is highly expressed in both primary and metastatic USC. Likewise, high p53 expression was observed in primary and metastatic USC tumours. Furthermore, we observed a significant negative correlation between p53 protein expression and survival. Thus, 5-years survival was 12% in patients with strong p53 staining compared to 60% in patients with negative to moderate expression. Western blot analysis revealed that USPC-1 cells express high levels of IGF-IR protein and mRNA, compared to USPC-2 cells. Furthermore, USPC-1 cells express high levels of p53 whereas USPC-2 cells express low p53 levels. Results of co-transfection assays revealed that expression of wild-type p53 in USPC-2 cells repressed IGF-IR promoter activity by ~65%. p53 mutated, however, was unable to inhibit promoter activity.

In addition, we measured the expression of zinc finger transcription factor Sp1 in USC cell lines. The rationale for these measurements was the fact that previous studies indicated that the mechanism of action of p53, at least in the context of IGF-IR gene regulation, involves physical and functional interactions with this ubiquitous nuclear protein.[32] Results of Western blots showed that the pattern of expression of Sp1 was diametrically opposite to that of p53. Thus, USPC-1 cells express low Sp1 levels whereas USPC-2 cells contain high Sp1 concentrations. To address the functional interactions between p53 and Sp1 in regulation of the IGF-IR gene, transient co-transfection assays were performed in USPC-2 cells using p53 and Sp1 expression vectors. The results obtained indicate that Sp1 induced a ~25% stimulation of IGF-IR promoter activity, while p53 suppressed ~60% of the Sp1-induced transactivation. Moreover, addition of Sp1-family binding inhibitor Mithramycin A, decreased IGF-IR promoter activity and endogenous IGF-IR protein levels. Finally, results of co-immunoprecipitation experiments showed that p53 action was associated with physical interaction with Sp1 protein. Interestingly, results of DNA affinity chromatography and chromatin immunoprecipitation assays identified the co-presence of both transcription factors in IGF-IR promoter DNA.[28]

The p53 pathway is activated in response to an extensive variety of cellular stress signals. These insults include DNA damage and telomere shortening, hypoxia, low nucleoside triphosphate pool sizes, spindle damage, heat and cold shock, inflammation and nitric oxide production, as well as activation of oncogenes by mutations.[39] These stresses have the potential to decrease the fidelity of cell cycle progression and DNA replication and, thus, to increase the mutation rates in cells.[40] Ligand-induced activation of the IGF-IR tyrosine kinase domain results in the recruitment and activation of PI3K to the plasma membrane receptor and activation of the Akt protein kinase. Akt has several antiapoptotic substrates, such as BAD[41] and Mdm2.[42] Akt is also translocated to the cell nucleus where it phosphorylates the forkhead transcription factors, leading to antiapoptotic signalling and cell growth.[43] PI3K activity is counteracted by PTEN, a lipid phosphatase. PTEN is a tumour suppressor that is frequently mutated in sporadic malignancies.[44] Association between the p53 and IGF-I pathways can occur at several levels. Thus, p53 was shown to regulate PTEN expression[45] while PTEN and inhibitors of Akt signalling upregulated p53 expression.[46] The ubiquitin ligase Mdm2 is of primary importance in regulation of p53 activity.[47] IGF-I was shown to induce p53 degradation in an Mdm2-dependent manner[48] while Mdm2 physically associates with IGF-IR and causes IGF-IR ubiquitination and degradation.[49] Various other mechanisms and pathways have been suggested to participate in the converging tasks of p53 and the IGF system[50] and are thus considered as potential therapeutic targets.[51]

IGF-IR targeting emerged in recent years as a promising therapeutic approach in a number of malignancies.[20,23,24] In the context of endometrial cancer, we have recently evaluated the ability of cixutumumab to inhibit proliferation and induce apoptosis. Our results showed that cixutumumab abrogated the IGF-I-stimulated increase in proliferation rate, and increased caspase-3 and PARP cleavage, two markers of apoptosis.[52] Identification of biomarkers that can predict a positive response to these therapies is of major clinical importance. The relevance of IGF-IR level as a prognostic and/or predictive factor has been a controversial issue for many years. In breast cancer, for example, IGF-IR is differentially expressed with variable prognostic impact among breast cancer subtypes.[53] No such information is yet available regarding endometrial cancer. Clearly, additional factors and signalling pathways must be taken into consideration when evaluating the potential benefit of anti-IGF-IR therapies, including the activation (phosphorylation) status of the IGF-IR and downstream mediators, such as IRS-1, *ras-raf*-MAPK, PKB/Akt, and others. Our study shows that USC tumours overexpressing p53 are more likely to benefit from anti-IGF-IR therapies. We postulate that enhanced IGF-IR expression in these cells is secondary to the aberrant p53 expression detected in these cells. Obviously, we cannot discard the involvement of additional tumour suppressors and transcription factors in control of IGF-IR gene expression in USC.

In summary, we provide evidence that p53 regulates IGF-IR gene expression in USC cells *via* a mechanism that involves repression of the IGF-IR promoter. Furthermore, our results demonstrate that the mechanism of action of p53 involves functional and physical interactions between p53 and Sp1 proteins. The interplay between the p53 and IGF-I signalling pathways is of major basic and translational relevance. Finally, the potential role of p53 as a biomarker for IGF-IR-directed therapies in USC (and, most probably, other types of cancer) must be confirmed by larger cell-based and patients analyses.

Conflict of interest statement

None declared.

Acknowledgements

This work was performed in partial fulfilment of the requirements for a Ph.D. degree by Zohar Attias-Geva in the Sackler Faculty of Medicine, Tel Aviv University. We thank Drs. A.D. Santin, E. Mercer and R. Tjian for cell lines and reagents. The authors wish to thank the Israel Cancer Research Fund (ICRF, Montreal, Canada) for their generous support.

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