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Antisense treatment of IGF-IR enhances chemosensitivity in squamous cell carcinomas of the head and neck

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ABSTRACT

To evaluate whether targeting IGF-IR therapeutic can increased chemosensitivity of squamous cell carcinomas of the head and neck (SCCHN) to doxorubicin and cisplatin, Insulin-like growth factor type I receptor (IGF-IR) expression was down-regulated by treatment with AS[S]ODN. Different doses of AS[S]ODN with doxorubicin or cisplatin were tested in TU159 and 183A SCCHN cell lines. Compared to phosphorothioate sense oligonucleotides (SS[S]ODN), AS[S]ODN treatment inhibited cancer cell proliferation and attenuated activation of IGF-IR. AS[S]ODN treatment was shown to enhance the sensitivity of SCCHN cell lines to doxorubicin and cisplatin. This observation correlated closely with the induction of apoptosis as measured by Annexin-V/PI and caspase activation assays. The *in vivo* results showed that treatment with AS[S]ODN/doxorubicin in combination also resulted in a significant suppression in TU159 xenografts. In conclusion, this study provides evidence for the efficacy of IGF-IR down-regulation combined with chemotherapy and raises the possibility that SCCHN treatment may be improved by pharmaceutical strategies directed towards the IGF-IR.

1. Introduction

Squamous cell carcinomas of the head and neck (SCCHN) arises in the mucosal lining of the upper aero-digestive tract and is the tenth most common cancer in men worldwide.[1] Despite advances in current therapies, the 5-year mortality rate of SCCHN patients has not improved in the past few decades. To discover novel and effective molecular-targeted approaches, the molecular mechanisms that lead to a poorly differentiated phenotype in SCCHN must be better understood.

Insulin-like growth factor I receptor (IGF-IR) is an attractive anti-tumour target for cancer therapy. Preclinical research have shown that the IGF-IR and its ligands IGF-I/II play important roles in the development and progression of human cancer.[2,3] After IGF stimulation, the intrinsic tyrosine kinase of the β-subunit becomes activated and phosphorylates a series of adaptor proteins, such as insulin receptor substrate-1 (IRS-1), to activate intracellular signalling pathways. High expression of IGFs and IGF-IR has also been associated with tumour metastatic potential[4] and has been found in a variety of human malignancies such as non-small-cell lung cancer and gastrointestinal stromal tumours.[5,6] Previous studies have demonstrated that IGF-IR expression is increased in human head and neck cancer and that IGF-IR signalling significantly enhances the proliferation, motility and tumourigenicity of

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human head and neck cancer cell lines.[7,8] However, the importance of IGF-IR overexpression with regard to therapy in human head and neck cancer is still unclear. In this study, we attempt to investigate whether IGF-IR down-regulation results in an enhanced chemosensitive phenotype of head and neck cancer cells.

2. Materials and methods

2.1. Cell cultures and transfection

IGF-IR phosphorothioate antisense oligodeoxynucleotide (5′-TCC TCC GGA GCC AGA CTT), (AS[S]ODN) and phosphorothioate sense oligodeoxynucleotides (SS[S]ODN) (5′-AAG TCT GGC TCC GGA GGA) were used as controls[9]; and phosphorothioate oligodeoxynucleotides ([S]ODN) was purchased from Biognostik (Goettingen, Germany). For [S]ODN transfection, cells were seeded at a density of 5000 cells/well in 96-well plates for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or 1.5×10^4 cells/well in 6-well plates for reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. When cells had grown to 50–60% confluency, transfection was carried out with Lipofectin reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After a 6-h transfection at 37 °C, the medium was replaced and incubated for an additional 72 h at 37 °C before further assays were carried out.

2.2. Reverse-transcription and real-time PCR

To quantify the levels of IGF-IR mRNA expression, real-time PCR was performed by using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) and the Opticon CFD-3200 Thermocycler (MJ Research) according to manufacturer's protocol. Previously described primers were used for these reactions. The primers for *IGF-IR* were 5′-GGG AAT GAA GTG CTG TAT G-3′ (forward) and 5′-CAC AGA AGC TTC GTT GAG AA-3′ (reverse). For amplification of human *GAPDH*, which serves as an internal control, the primers were 5′-GGACCTGACTGAC-TACCTC-3′ (forward) and 5′-CATACTCCTGCTTGCTG-3′ (reverse). The number of copies of IGF-IR transcripts was normalised to the number of copies of the housekeeping gene *GAPDH*.

2.3. Cell growth assays

Human head and neck cancer cell lines 183A and TU159 (Rockville, MD, USA) were grown in DMEM media with 10% foetal calf serum V/V at 37 °C with 5% CO_2 V/V. Exponentially growing 183A and TU159 cells (3×10^3 cells/well) were treated with different concentrations of IGF-IR [S]ODNs for 72 h. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, USA) was used to measure cell viability. For the combined treatment assay, cells (3×10^3 cells/well) were treated with IGF-IR antisense oligonucleotides (1.6 μM) for 24 h followed by treatment with cisplatin or doxorubicin in the concentrations indicated. After a further incubation time of 48 h, cell viability was quantified using the MTT assay.

2.4. Western blot analysis

Western blot analysis was performed according to the standard protocol, using antibodies against IGF-IRβ (at a 1:500 dilution), phospho-IGF-IR (at a 1:500 dilution), p-erk1/2 (at a 1:1000 dilution), erk1/2 (at a 1:500 dilution), pAkt (at a 1:500 dilution), Akt (at a 1:1000 dilution), cyclinD1 (at a 1:500 dilution), bcl-2 (at a 1:200 dilution), p27 at a 1:500 dilution (Santa Cruz Biotechnology), IRS-1 (at a 1:1000 dilution), p-IRS-1 at a 1:500 dilution (Epitomics) and PARP at a 1:500 dilution (Pharmigen).

2.5. Vascular endothelial growth factor expression (VEGF)

To determine changes in VEGF expression in human cell culture, we used an ELISA kit specific to human VEGF-A (BioSource Europe, Nivelles, Belgium). Cells were plated at 40–50% density and incubated with or without [S]ODN for 72 h. The culture medium was then tested for VEGF using a commercial ELISA (R&D Systems, Minneapolis, MN).

2.6. Apoptosis assay

After 24-h pretreatment with IGF-IR antisense oligonucleotides (1.6 μM), TU159 cells (3×10^3 cells/well) were treated with doxorubicin in the concentrations indicated; a total of 1×10^6 TU159 cells were washed twice with ice-cold PBS before simultaneous addition of fluorescein isothiocyanate (FITC) labelled Annexin-V and propidium iodide (BD Biosciences). The final mixture was incubated for 15 min at room temperature in the dark and then analysed by flow cytometry to determine the percentage of apoptotic cells.

2.7. Caspase activity assay

Caspase-3 activities were measured using a commercially available kit according to the manufacturer's instructions.[10] Briefly, cells were suspended in 50 μl of lysis buffer and incubated on ice for 10 min. The cells were then centrifuged for 1 min in a microcentrifuge (10,000) and the supernatants were collected for the assay. For each assay, 50–200 μg of protein was added to 50 μl of cell lysis buffer, followed by addition of 50 μl of 2X reaction buffer and 5 μl of 4 mM DEVD-pNA (caspase-3). The mixture was incubated at 37 °C for 1–2 h and read at 405 nm in a microtitre plate reader.

2.8. In vivo tumour study

TU159 cell xenografts were generated by the injection of 3×10^6 cells in the flanks of the severe combined immunodeficient (SCID) mice. Treatment started when tumours were 100–200 mm^3. The mice were randomised into four groups of eight animals each and were given i.p. injections of AS[S]ODN (100 mg/kg), doxorubicin (1 mg/kg) or doxorubicin + AS[S]ODN (100 and 1 mg/kg, respectively) three times a week for 3 weeks. At each treatment, tumour volume was measured with calipers (volume = L × w × w × 0.52) and the percentage of tumour growth inhibition was calculated as Inhibitory rate (%) = (Vcontrol − Vtreatment)/Wcontrol × 100.

2.9. Statistical analysis

Statistical analysis was performed using SPSS software (Release 11.0, SPSS Inc.). Data were expressed as the mean ± SD. Results were considered significant if $p < 0.05$, as obtained by an appropriate ANOVA procedure and Student's t-test.

3. Results

3.1. Antisense oligonucleotide-mediated down-regulation of IGF-IR mRNA and protein expression

The effects of IGF-IR AS[S]ODN on mRNA and protein expression were assessed by real-time PCR and Western blotting, respectively (Fig. 1A). In TU159 and 183A cells, the mRNA and protein levels of IGF-IR expression were significantly reduced after the addition of 1.6 μM AS[S]ODN compared to untreated cells ($p < 0.01$). Treatment with S[S]ODN had only marginal effects on IGF-IR expression. These results indicate that treatment with AS[S]ODN against IGF-IR specifically inhibited IGF-IR expression.

3.2. Effect of AS[S]ODN on IGF-IR-mediated signalling in SCCHN cells

Cells were incubated for 48 h with various doses of AS[S]ODN, starved for 24 h and subsequently stimulated with IGF-I for 10 min. Western blot analysis shows that inhibition of IGF-IR and IRS-1 substrate with AS[S]ODN was dose-dependent, and ultimately yields a marked reduction in phosphorylation of downstream signalling pathways (Fig. 2A). To characterise AS[S]ODN effects on the cell cycle, we performed additional Western blot experiments to elucidate underlying molecular mechanisms. TU159 and 183A cells were treated with different concentrations of AS[S]ODN for up to 72 h and resulted in a dose-dependent suppression of cyclin D1, which is essential for the transition from G1 to S-phase. At the same time, expression of the cyclin-dependent kinase inhibitors p27Kip1 markedly increased (Fig. 2B). Since the IGF-IR system has been shown to be important for regulating VEGF expression in cancer cells, the impact of AS[S]ODN on VEGF secretion was measured by ELISA. The treatment of SCCHN cells with AS[S]ODN leads to a substantial reduction in VEGF secretion

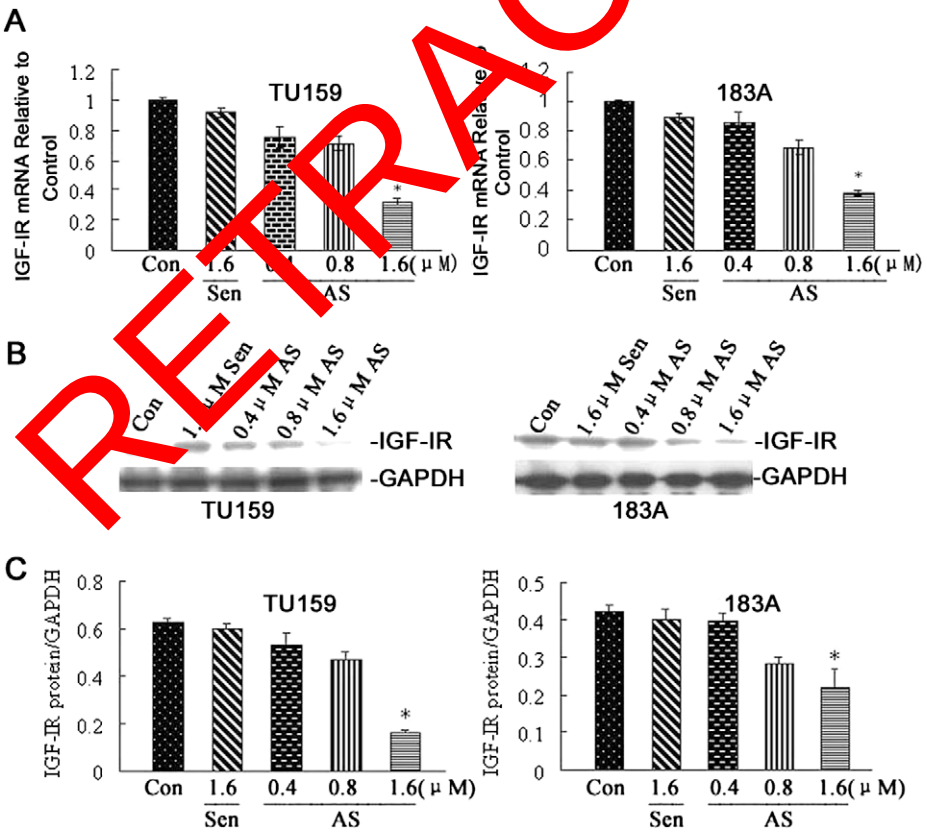

Fig. 1 – Insulin-like growth factor I receptor (IGF-IR) mRNA and protein expression in squamous cell carcinomas of the head and neck (SCCHN) cells treated with IGF-IR phosphorothioate oligodeoxynucleotides ([S]ODN). The human cancer cells were treated with indicated doses of IGF-IR phosphorothioate antisense oligonucleotides (AS) or phosphorothioate sense oligonucleotides (Sen) for 72 h. IGF-IR mRNA (A) and protein expression (B and C) were determined by real-time PCR and western-blot; the immune slot blot was scanned and the ratio of IGF-IR protein to GAPDH was calculated. Values represent the mean (columns) ± SD ($n = 3$). *$P < 0.01$ compared to control.

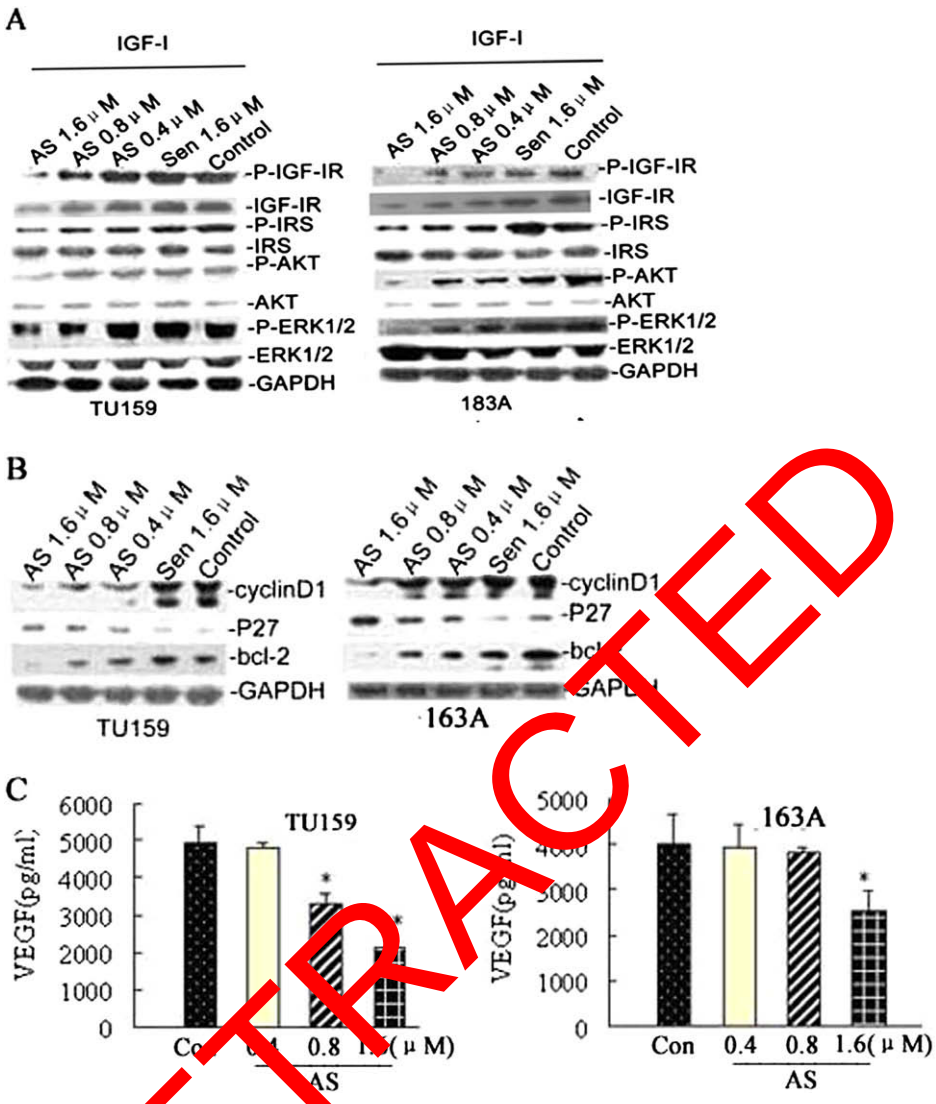

Fig. 2 – Effect of IGF-IR [S]ODN treatment on signalling pathway activation in SCCHN cells. (A) Cancer cells were incubated with the indicated doses of phosphorothioate antisense oligonucleotides (AS) or phosphorothioate sense oligonucleotides (Sen) for 48 h, starved for 24 h and subsequently stimulated with IGF-I (50 ng/ml) for 15 min. The effects on IGF-IR down-stream signalling pathways were determined by Western blot. (B) Cancer cells were incubated in complete media with the indicated concentrations of AS or Sen for 72 h. P27, cyclinD1 and bcl-2 were tested by Western blot. (C) Cancer cells were treated with the indicated doses of AS or Sen for 72 h and the supernatants were analysed for vascular endothelial growth factor expression (VEGF) by ELISA. Mean (columns) ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$ compared to control.

(Fig. 2C, $p < 0.01$). This decrease in VEGF was also confirmed on an mRNA level by real-time PCR (data not shown).

3.3. *IGF-IR antisense treatment suppresses SCCHN cell proliferation and sensitises SCCHN cells to chemotherapy*

Treatment of TU159 and 183A cells with IGF-IR antisense oligonucleotides resulted in a dose-dependent decrease in cellular proliferation compared to untreated cells, as determined by the MTT assay (Fig. 3A). No significant differences in cellular proliferation were detected between untreated or sense oligonucleotide-treated cells. Prior work on combination therapy of cytotoxic drugs with IGF-IR-inhibition by antisense oligonucleotide demonstrated encouraging results in other tumour entities.[11] Based on this, we investigated whether IGF-IR down-regulation enhances the chemosensitivity of TU159 and 183A cells. Cells were pretreated with IGF-IR AS[S]ODN before adding increasing concentrations of either doxorubicin or cisplatin. The cells were subsequently analysed by the MTT assay and the chemosensitivity of IGF-IR AS[S]ODN-treated cells was compared to S[S]ODN control cells. IGF-IR down-regulation by AS[S]ODN in TU159 cells resulted in a significant increase in chemosensitivity to doxorubicin but moderate to cisplatin (Fig. 3B). When these cells were treated with doxorubicin alone or in combination with AS[S]ODN, the IC_{50} values were 0.23 and 0.01 μg/ml, respectively ($p < 0.001$). Compared with cisplatin alone, the effect of combination treatment

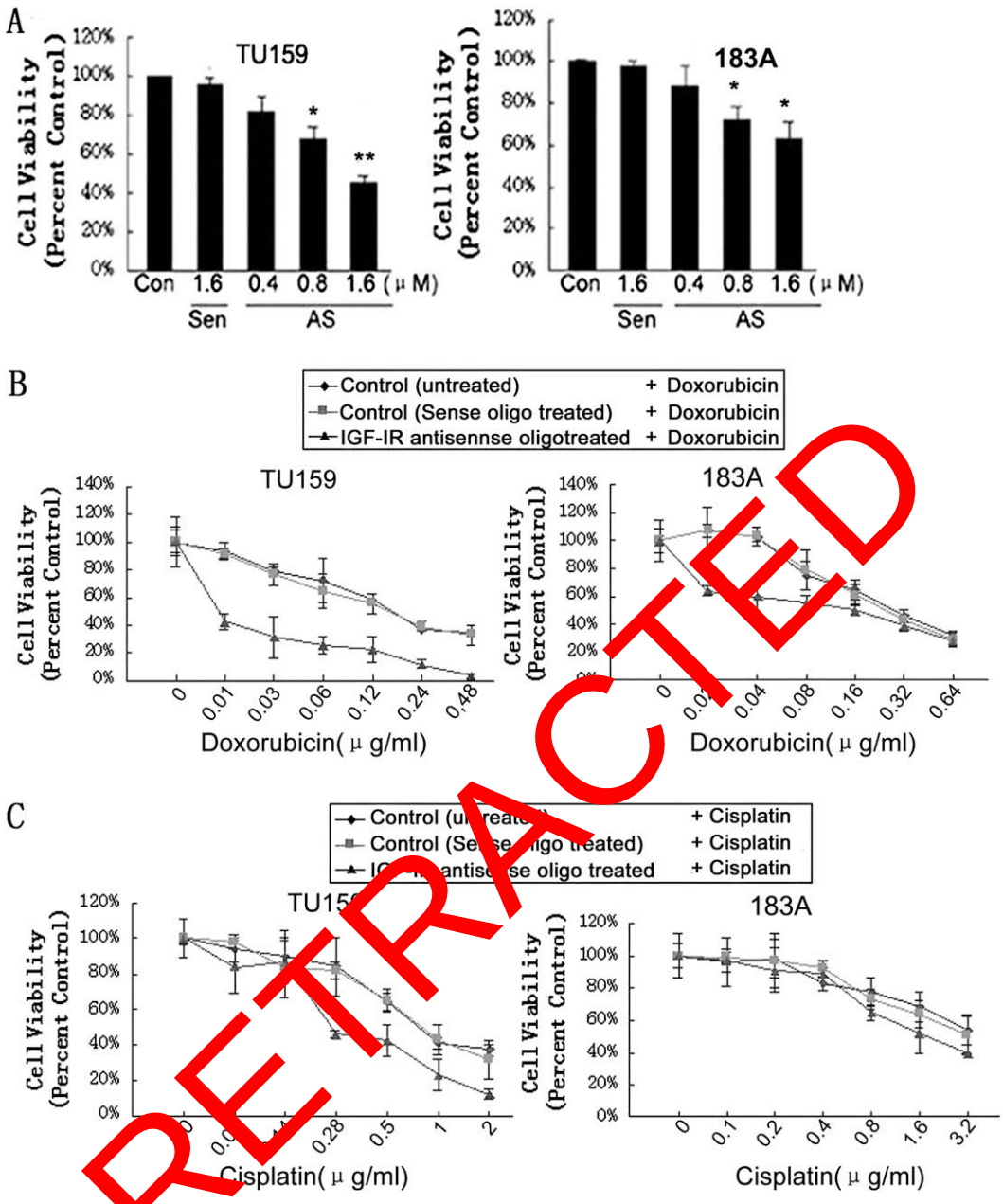

Fig. 3 – IGF-IR [S]ODN treatment suppresses SCCHN cells proliferation and sensitises cells to chemotherapy. Cells were treated for 72 h with the indicated doses of phosphorothioate antisense oligonucleotides (AS) or phosphorothioate sense oligonucleotides (Sen). (A) Cell viability was measured by MTT assay. Values represent the absorbance at 490 nm. *P < 0.05, **P < 0.01 compared to control. Down-regulation of IGF-IR through AS treatment results in significantly increased chemosensitivity of TU159 and 183A cells to doxorubicin (B) and cisplatin (C). Values represent the mean percentage of survival (±SD, n = 4) compared to control which was not drug treated.

with both AS[S]ODN and cisplatin was more apparent (IC_{50} values were 1.24 and 0.26 μg/ml, respectively, $p < 0.01$). However, the increased sensitivity of 183A cells to doxorubicin or cisplatin by IGF-IR down-regulation was less apparent. For these cells, the IC_{50} values of doxorubicin with and without AS[S]ODN were 0.31 and 0.60 μg/ml, respectively ($p < 0.05$). The IC_{50} for treatment with cisplatin alone or in combination of AS[S]ODN were 3.15 and 1.5 μg/ml, respectively ($p < 0.05$).

3.4. *IGF-IR AS[S]ODN enhances the induction of apoptosis by doxorubicin*

The MTT assay provided evidence that AS[S]ODN treatment enhanced the sensitivity of SCCHN cells to doxorubicin, but it does not provide additional details behind the mechanism of cell death. To determine whether the increased chemosensitivity reflected an induction of apoptosis, Annexin-V/PI staining and flow cytometric analysis were performed to

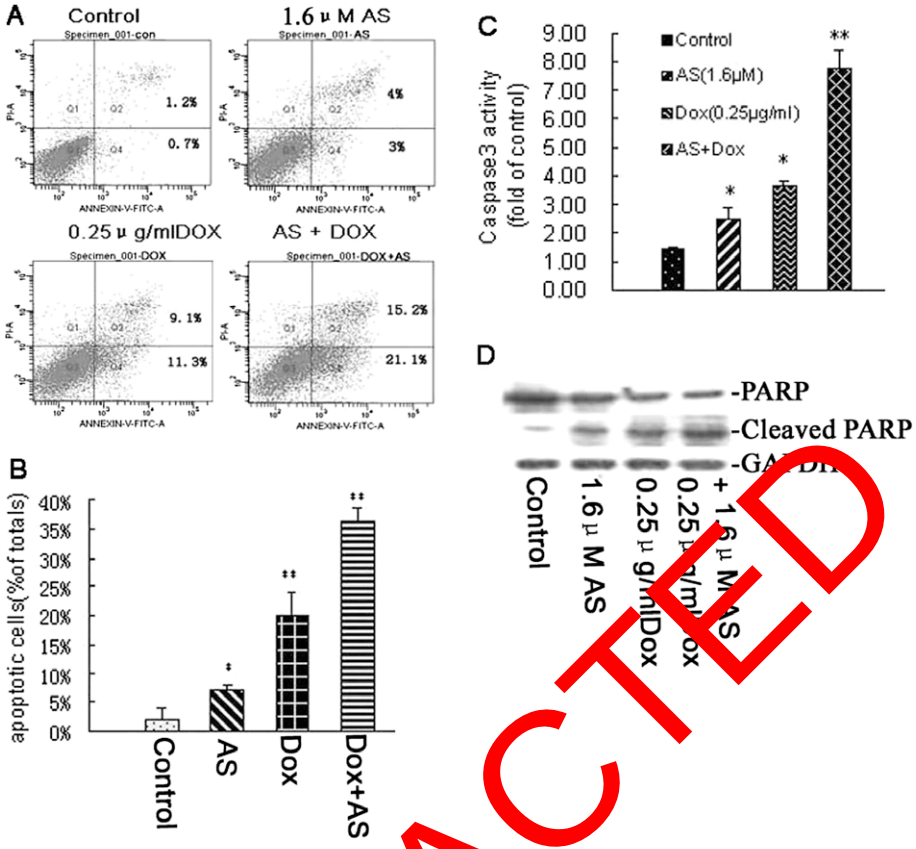

Fig. 4 – Phosphorothioate antisense oligonucleotides (AS) enhance the apoptosis induced by doxorubicin in TU159 cells. After 24 h pretreatment with IGF-IR AS, cells were further treated with or without doxorubicin (DOX) for 48 h. (A) Cell apoptosis analysed by flow cytometry. (B) Apoptosis estimated by flow cytometry assay in TU159 cells after 48 h treatment with either AS, doxorubicin or their combination. (C) Caspase-3 activities were measured as described. (D) Western blot was performed to assess poly ADP-ribose polymerase (PARP) cleavage. Mean (columns) ± SD (bars). *P < 0.05, **P < 0.01, statistically significant in comparison with control.

detect apoptotic cells. Treatment of TU159 cells with AS[S]ODN and/or doxorubicin resulted in an increase of apoptotic cells (Fig. 4A and B). Although single-agent doxorubicin also induced apoptosis in TU159 cells ($P < 0.05$), the number of apoptotic cells was significantly increased upon addition of antisense IGF-IR ($P < 0.01$). These results were confirmed by a caspase-3 activity assay and by proteolytic processing of poly ADP-ribose polymerase (PARP), which is involved in DNA repair (predominantly with response to environmental stress). Our study found that the activity of caspase-3 in TU159 cells treated with both AS[S]ODN and doxorubicin increased 6.28-fold compared to that in control. The activity after treatment with AS[S]ODN or doxorubicin alone, however, increased only 2.50 and 2.13-fold higher than the control (Fig. 4C). For these cells, doxorubicin treatment induces cleaved-PARP, while the combination of doxorubicin and AS[S]ODN induces cleaved-PARP significantly (Fig. 4D). These results indicate that IGF-IR signalling blockade combined with doxorubicin promotes caspase-mediated apoptosis in TU159 cells.

3.5. *IGF-IR AS[S]ODN and doxorubicin in tumour xenografts*

SCID mice bearing TU159 xenografts were treated with these drugs alone or in combination using concentrations as described in Section 2. By the 4th week of treatment, statistically significant differences in tumour volume were noted between all groups (Fig. 5A). An incremental reduction in tumour volume compared with controls was noted for all groups receiving treatment: AS[S]ODN, doxorubicin or AS[S]ODN combined with doxorubicin. At the conclusion of treatment, animals receiving both AS[S]ODN and doxorubicin harboured tumours 2.9-fold smaller than controls ($P < 0.01$). Tumours from animals receiving either AS[S]ODN or doxorubicin alone were 1.6-fold ($P < 0.05$) and 2.1-fold ($P < 0.01$) smaller than those from controls, respectively. These data indicate that AS[S]ODN and doxorubicin displayed significantly higher anti-tumour activity than either agent alone. In order to investigate whether the anti-tumour activity of AS[S]ODN was associated with the down-regulation of IGF-IR, IGF-IR levels in AS[S]ODN treatment

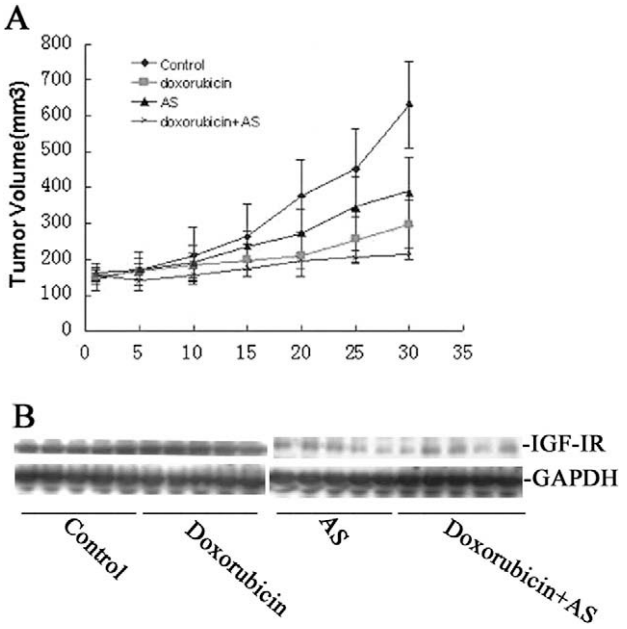

Fig. 5 – Inhibition of SCCHN xenograft growth by IGF-IR phosphorothioate antisense oligonucleotides (AS) and doxorubicin. (A) TU159 cells (3×10^6) s.c. injected in each flank of athymic nude mice. Mice with established tumours (100–200 mm^3) were injected i.p. with AS, doxorubicin or AS and doxorubicin in combination as described in Section 2 at thrice a week for 2 weeks. Each point represents the mean volume ± SD of 8 independent tumours. (B) Five samples were used to analyse the expression of IGF-IR in each group; each tumour lysate (1 mg) was separated by a 10% SDS-PAGE and transferred to nitrocellulose blots. The blots were probed with anti-IGF-IR antibody.

of TU159 tumours were tested by Western blot. The results show that the IGF-IR level was lower in AS[S]ODN treatment animals than the IGF-IR in untreated animals (Fig. 5B).

4. Discussion

IGF-IR is overexpressed in a variety of malignant human tumours, including squamous cell carcinomas of the head and neck (SCCHN).[7,8] It is widely accepted that the IGF-axis activates anti-apoptotic signalling, which in turn upregulates the PI3K and MAPK pathways in cancer cells.[12] Inhibition of IGF-IR signalling has been proposed as a potential means of optimising anticancer therapy.[13] In addition to its roles in transformation and survival, the IGF/IGF-IR system also regulates vascular endothelial growth factor production, suggesting a role in angiogenesis.[14] These factors nominate IGF-IR as an attractive anticancer target.

In our study, we attempted to use phosphorothioate antisense oligonucleotides to silence endogenous IGF-IR expression and explored the effects of IGF-IR down-regulation in head and neck cancer cells. We were able to demonstrate that IGF-IR mRNA and protein expression were significantly reduced in antisense oligonucleotide-treated cells as compared to those in control cells. IGF-IR down-regulation in TU159/183A cells resulted in a dose-dependent decrease in cellular proliferation, induction of apoptosis, caspase activation and reduced expression of pro-angiogenic cytokines such as VEGF. These results are in line with previous findings *in vitro* of antisense oligonucleotide-mediated IGF-IR down-regulation in human lung cancer cells,[15] prostate cancer cells[16] and malignant glioma cells.[17] Since the IGF/IGF-IR pathway plays an important role in cell growth and proliferation, we investigated whether IGF-IR AS[S]ODN could interfere with the activation of signalling pathways in SCCHN cells. Western blot analysis showed that blocking IGF-IR with AS[S]ODN resulted in a dose-dependent inhibition of IGF-IR and IRS-1 substrate as well as brought about a marked reduction in phosphorylation of downstream signalling pathways. Results have shown that IGF-IR signalling prevents tumour cells from the cytotoxic effects of chemotherapy and may play an important role in cancer cell drug resistance.[18] Consequently, blocking of IGF-IR signalling has led to the enhancement of the chemosensitivity, making it a logical target for therapeutic intervention.[19] The present study discovered that combined treatment with IGF-IR AS[S]ODN and doxorubicin significantly enhances the cytotoxic effects of chemotherapeutic drugs *in vitro* and in TU159 xenograft, but the combination of oligonucleotides with cisplatin is poorly efficient when compared to doxorubicin to reduce cell viability. This may be related to the different action mechanism between doxorubicin and cisplatin. Most anticancer agents, including doxorubicin and cisplatin, killing cancer cells by inhibiting cell cycle and/or inducing apoptosis,[20–22] the mechanisms underlying apoptosis depended on the drug used. Such as in hepatocellular carcinomas HepG2 cells, cisplatin up-regulates *p53* and *p21* gene expression, whereas induction of apoptosis by doxorubicin is concomitant with down-regulated *p21* gene expression.[23,24]

Our results indicated that the combination of oligonucleotides with chemotherapeutic drugs might offer an alternative treatment strategy against squamous cell carcinomas of the head and neck. A number of authors have similarly reported that IGF-IR signalling results in resistance to chemotherapy in a wide variety of tumours.[25] The induction of apoptosis by chemotherapeutic drugs has been extensively studied and is thought to be the main mechanism of action. However, ligand-activated IGF-IR is emerging as a powerful inhibitor of apoptosis induced by a variety of agents, including anticancer drugs and ionising radiation.[26,27] In particular, Dunn and colleagues.[28] first showed that IGF-IR could induce a 20–40% increase in survival of breast cancer cells treated with clinically relevant and functionally diverse anticancer drugs. This work supports the idea that IGF-IR activation may significantly decrease the effectiveness of chemotherapy. Therefore, we treated SCCHN cell lines with IGF-IR AS[S]ODN in an attempt to further sensitise these cells to doxorubicin or cisplatin treatment. We observed that AS[S]ODN does sensitises SCCHN cell lines to treatment with these drugs via induction of apoptosis, as shown by an increase in cleaved poly (ADP-ribose) polymerase and caspase-3. IGF-IR targeting offers the potential advantage that, in addition to regulating apoptosis susceptibility, the IGF pathway mediates other aspects of malignant phenotype, including tumour cell proliferation, motility, adhesion and response to DNA damage.[29–32]

Our results clearly demonstrate that IGF-IR antisense treatment suppresses SCCHN cell proliferation and sensitises SCCHN cells to chemotherapy. The combination of IGF-IR antisense oligonucleotides with chemotherapeutic agents may serve as a novel approach for the treatment of SCCHN.

Conflict of interest statement

None declared.

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