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Interferon-λ induces G1 phase arrest or apoptosis in oesophageal carcinoma cells and produces anti-tumour effects in combination with anti-cancer agents

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ABSTRACT

Signal pathways of novel type III interferons (IFN-λs) are similar to those of type I IFNs (IFN-α/β) but their distinct functions have not been well characterised. We examined the growth suppressive activity of IFN-λ1 with nine human oesophageal carcinoma cell lines expressing the IFN-λ receptor complexes. Among them, three lines but not others showed IFN-λ1-mediated growth suppression by inducing G1 phase arrest or apoptosis. The G1 phase arrest was accompanied by the up-regulation of p21 and dephosphorylation of retinoblastoma (Rb), and the apoptosis was evidenced by cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP). Similar but not identical susceptibility was found in IFN-α-treated oesophageal carcinoma cells. Despite the differential suppressive responses among the cells, all the cells increased the expression of the *myxovirus resistance* A (MxA) and *2′,5′-oligoadenylate synthetase* (2′,5′-OAS) genes and class I antigens of the major histocompatibility complexes (MHC) with IFN-λ1 treatment. Fibroblasts and mesenchymal stem cells, positive for IFN-α receptor (IFNAR), lacked one of the IFN-λ receptor complexes and Het-1A, immortalised oesophageal epithelium cells, were insensible to the IFN-λ1-induced growth suppression. IFN-λ1 produced combinatory anti-tumour effects with chemotherapeutic agents, cisplatin (CDDP) and 5-fluorouracil (5-FU), in IFN-λ1-sensitive oesophageal carcinoma cells but not in normal or Het-1A cells, while IFN-α achieved the combinatory suppressive effects to normal cells. These data collectively show that IFN-λ1 responsiveness is tissue-specific due to the restricted receptors expression and is diversified even among cells of the same lineage, and suggest that IFN-λ1 is a potential therapeutic agent for oesophageal carcinoma without damaging surrounding tissues.

1. Introduction

Recently identified IFN-λs, consisting of IFN-λ1, -λ2 and -λ3, belong to type III IFN and are also known as interleukins (IL)-29, -28A and -28B, respectively.[1,2] All the IFN-λs bind the same heterodimeric receptor complexes composed of the IL-10 receptor β (IL-10Rβ) and a novel IL-28 receptor α (IL-28Rα).[1,2] Ligation of the receptor complexes induces acti-

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vation of the Janus kinases and the phosphorylation of STAT1 and STAT2, and results in the formation of the IFN-stimulated regulatory factor 3 together with IFN-regulatory factor 9. The multi-factor complex, which is also induced by type I IFNs, binds to the IFN-stimulated response elements and initiates IFN-responsible transcriptions.[1] Biological activities of IFN-λs therefore can overlap with those of type I IFNs but the possible distinctive functions of IFN-λs remain unclear.

Type I IFNs are pleiotropic cytokines with anti-viral and immunomodulatory effects. Type I IFNs can up-regulate the expression of class I molecules of MHC and induce anti-viral molecules such as MxA and 2′,5′-OAS.[3,4] Likewise, IFN-λs increase the MHC class I expression and induce the anti-viral molecules.[1,5,6] IFN-λs showed the same anti-virus repertoires as type I IFNs but precise analyses suggest differential activation manners in the signalling processes between type I and type III IFNs.[7] In contrast immunological effects of IFN-λs have been less characterised but several studies showed that IFN-λs were produced in dendritic cells upon stimulation with the toll-like receptors and could induce proliferation of regulatory T cells.[8,9] Moreover, expression of the *IFN-λ* gene in tumours achieved anti-tumour effects against the tumours by activating natural killer and cytotoxic T cells, suggesting that IFN-λs are involved in innate and acquired immunity.[10,11] Type I IFNs have an anti-proliferative property to various cell types primarily through inducing apoptosis.[3,4] The apoptosis is favourable to hosts by eliminating virus-infected cells and also contributes to direct anti-tumour effects. The possible growth inhibitory effects of IFN-λs however have not been well investigated. Several studies reported IFN-λ-mediated growth suppression *in vitro* in a neuroendocrine tumour,[12] a glioblastoma,[13] and several human colon tumour cell lines.[5] Detailed mechanisms of the IFN-λs-mediated growth inhibition however were not well characterised and in particular the growth suppression has not been tested in a number of cells of the same tissue origin. Moreover, a possible combination with anti-cancer agents has not been examined.

IL-28Rα determines specificity of the ligand binding as well as recruitment of intracellular signalling molecules.[1,2,14] Two alternatively spliced variants of IL-28Rα have been identified in human,[2] which have either 29 amino acids deletion in its intracytoplasmic part or only the ectodomain. Type I IFNs receptors, IFNAR1 and IFNRA2, are expressed in most of nucleated cells and consequently type I IFNs have a broad range of the targets irrespective of cell types. The initial reports demonstrated that the IL-28Rα receptors were expressed in many kinds of tissues[2] but subsequent studies suggested the limited expression[15,16] in contrast to ubiquitously expressed IL-10Rβ.[15] Further investigations are required to delineate the target specificity of type III IFNs since the IL-28Rα expression determines the responsiveness to IFN-λs.[1,2,14]

Oesophageal carcinoma, frequently developed in aged persons, needs effective therapeutic strategies to improve the poor prognosis. Type I IFNs have been clinically examined in various types of malignancy including oesophageal carcinoma. Combinatory regimens with type I IFNs and anti-cancer agents have also been investigated in clinical settings[17] but type I IFNs produce many kinds of adverse effects.[18] In contrast, IFN-λs have not been examined for the anti-tumour effects in the combination with anti-cancer agents and none of clinical studies with IFN-λs have yet been conducted. In this study we examined whether IFN-λs could inhibit the proliferation of human oesophageal carcinoma cells and found that IFN-λ1 induced G1 phase arrest or apoptosis. We also investigated the expression of the IFN-λs receptors in normal cells and tested possible combinatory effects with IFN-λ1 and 5-FU or CDDP, which are commonly used for oesophageal carcinoma treatment.

2. Materials and methods

2.1. Cell lines

Human oesophageal carcinoma TE-1, TE-2, TE-10, TE-11, YES-2, YES-4, YES-5, YES-6 and T.Tn cells, human mesothelioma NCI-H2452, NCI-H2052, NCI-H226, NCI-H28 and MSTO-211H cells, human pancreatic carcinoma BxPC-3, PANC-1, AsPC-1 and MIA-PaCa-2 cells, and human fibroblasts OUMS-24[19] and HFF cells were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS). Human mesenchymal stem cells (MSC) and human oesophageal epithelial Het-1A cells were purchased from Cambrex (Walkersville, MD, USA) and American Type Culture Collection (Manassas, VA, USA), respectively, and they were maintained with a specified medium as recommended by the suppliers.

2.2. Northern blot analysis

RNA (20 μg) was subjected to electrophoresis on a formaldehyde-agarose gel and transferred to a nylon filter. The filter was hybridized with [α-^{32}P] dCTP-labelled cDNA in a QuickHib solution (Stratagene, La Jolla, CA, USA) as described elsewhere.

2.3. Flow cytometry and cell cycle analysis

Cells were cultured with IFN-λ1 (100 ng/ml) for 3 days, stained with either fluorescein isothiocyanate (FITC)-conjugated anti-HLA-A,B,C antibody or FITC-conjugated isotype-matched control antibody (BD Biosciences, San Jose, CA, USA) and analysed for their fluorescence intensity with FACScan (BD Biosciences) and CellQuest software (BD Biosciences). For cell cycle analysis, cells were cultured with IFN-λ1 (100 ng/ml) in 10% FCS-containing medium. For synchronisation at G1 phase, cells were cultured with 0.1% FCS-containing medium for 2 days before IFN-λ1 (100 ng/ml) treatment. Cells were then fixed in ice-cold 70% ethanol, treated with RNase (50 μg/ml) and stained with propidium iodide (PI, 50 μg/ml). Cell cycle distribution was analysed with FACScan and CellQuest software.

2.4. In vitro cytotoxicity and cell proliferation

Cells were seeded into 96-well plates and treated with human recombinant IFN-λ1 (R&D, Minneapolis, MN, USA), recombinant IFN-α2a (Santa Cruz Biotech, Santa Cruz, CA, USA), 5-FU (Wako, Osaka, Japan) or CDDP (Wako) for 5–6 days. Viable cells were measured with a cell-counting WST kit (Dojindo, Kumamoto, Japan). The amount of formazan produced was

determined with the absorbance at 450 nm. Viable cell numbers after IFN-λ1 treatment were also determined with the trypan blue dye exclusion test.

2.5. Staining with Annexin V

Cells were cultured with IFN-λ1 (100 ng/ml) for 3 days. Adherent and non-adherent cells were reacted with FITC-conjugated Annexin V (BD Biosciences) and PI for 15 min. The staining profiles were determined with FACScan and CellQuest software.

2.6. Western blot analysis

Cells were treated with IFN-λ1 (100 ng/ml) for 3 days and lysed with a protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA). The cell lysates were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis and were probed with anti-p21 antibody (Ab) (Santa Cruz), anti-Kip1/p27 Ab (BD Biosciences), anti-phospho-Rb (at Ser795) Ab, anti-Rb Ab, anti-cleaved caspase-3 Ab, anti-caspase-3 Ab or anti-PARP Ab (Cell Signaling, Beverly, MA, USA). A pan-caspase inhibitor Z-VAD-FMK or Z-FA-FMK as a control (Calbiochem, Darmstadt, Germany) was used for 3 h before the IFN-λ1 treatment. The membranes were developed with the ECL system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and the same filter was re-probed with anti-actin Ab (Sigma–Aldrich) as a control. The intensity of the respective signals in these blots was determined with an image analysis using the public domain Image J program (available at http://rsb.info.nih.gov/ij).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Cells were treated with IFN-λ1 (100 ng/ml) or IFN-α2a (100 ng/ml) for 24 h and the total RNA was used to synthesize first-strand cDNA with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Amplification of equal amounts of the respective cDNA was performed with the following primers and conditions: for the *IFNAR1* gene, 5′-CTTTCA AGTTCAGTGGCTCCACGC-3′ (sense) and 5′-TCACAGGCGT GTTTCCAGACTG-3′ (anti-sense), and 10 s at 94 °C for denaturation/20 s at 60 °C for primer annealing/25 cycles for amplification; for the *IFNAR2* gene 5′-GAAGGTGGTTAAGAACTGTGC-3′ (sense) and 5′-CCCGCTGAATCCTTCTAGGACGG-3′ (anti-sense), and 10 s at 94 °C/20 s at 56 °C/25 cycles; for the *IL-28Rα* gene, 5′-GGGAACCAAGGAGCTGCTATG-3′ (sense) and 5′-TGGCACTGAGGCAGTGGTGTT-3′ (anti-sense), and 10 s at 94 °C/20 s at 58 °C/28 cycles; for the *IL-10Rβ* gene, 5′-TATTGGACCCCCTGGAAT-3′ (sense) and 5′-GTAAACGCACCACAGCAA-3′ (anti-sense), and 10 s at 94 °C/20 s at 50 °C/28 cycles; for the *MxA* gene, 5′-AGATCCAGGACCAGCTGAGCCTGT-3′ (sense) and 5′-GTGGAACTCGTGTCGGAGTCTGGTA-3′ (anti-sense), and 10 s at 94 °C/20 s at 62 °C/29 cycles; for the *2′,5′-OAS* gene, 5′-ATTGACAGTGCTGTTAACATCATC-3′ (sense) and 5′-AGATCAATGAGCCCTGCATAAACC-3′ (anti-sense), and 10 s at 94 °C/20 s at 55 °C/27 cycles; for the *p21* gene, 5′-GACACCACTGGAGGGTGACT-3′ (sense) and 5′-GGCGTTTGGAGT GGTAGAAA-3′ (anti-sense), and 10 s at 94 °C/20 s at 52 °C/30 cycles; for the *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene, 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (anti-sense), and 15 s at 94 °C/15 s at 60 °C/23 cycles.

3. Results

3.1. IFN-λ receptors expression and responsiveness to IFN-λ1 in oesophageal carcinoma cells

We examined the expression of the *IL-28Rα* and the *IL-10Rβ* genes in nine kinds of human oesophageal carcinoma cells (Fig. 1A). All the cells expressed the receptor genes with variable transcriptional levels. We also examined whether recombinant IFN-λ1 could up-regulate MHC class I molecules and induce the *MxA* and the *2′,5′-OAS* genes (Fig. 1B and C). We found that the IFN-λ1 treatment increased the class I expression level and activated the gene expressions although the enhanced levels of both protein and mRNA were variable among the cell lines. These data demonstrated that the oesophageal carcinoma cells were positive for the IFN-λ receptor complexes and the downstream signal pathways were functional; however, the responsiveness to the ligand was different among the cells.

3.2. IFN-λ1-mediated growth suppression

We examined whether IFN-λ1 could suppress growth of the oesophageal carcinoma cells (Fig. 2). We treated the cells with IFN-λ1 and examined the viability and the cell proliferation rates. Among the cells, TE-11, YES-5 and T.Tn cells were susceptible to IFN-λl-mediated growth suppression ($P < 0.01$ compared with the respective untreated cells at 10 ng/ml) in a dose-dependent manner, TE-1, YES-4 and YES-6 cells were insensitive (TE-1, $P = 0.21$; YES-4, $P = 0.34$; YES-6, $P = 0.88$ at 10 ng/ml) and TE-2, TE-10 and YES-2 cells were intermediated in the sensitivity (TE-2, $P = 0.046$; TE-10, $P = 0.77$; YES-2, $P = 0.011$ at 10 ng/ml) (Fig. 2A). The differential sensitivities to IFN-λ1 among the cell lines did not correlate with induced expression levels of the class I and anti-viral molecules. We also examined the suppressive activity of IFN-α and found that the suppressed growth in the respective cells was similar but not identical to IFN-λ1-mediated suppression since T.Tn cells were susceptible to IFN-λ1 but not to IFN-α (Fig. 2A and B). The dose-dependent suppression data showed that the activity of IFN-λ1 was relatively weak compared with IFN-α in most of the cells. We confirmed the IFN-λ-mediated suppressive activity with cell growth kinetics (Fig. 2C and D). Growth of the IFN-λ-sensitive cells was significantly retarded but the insensitive cells did not show any growth inhibition. These data imply that IFN-λ1- and IFN-α-induced growth suppression are dependent on the cell types and that the signal pathways of the IFN-λ1-induced growth inhibition are different from those of IFN-λ1-mediated up-regulation of the class I expression and the anti-viral molecules.

3.3. G1 phase arrest induced with IFN-λ1 treatment

We investigated cell cycle changes after IFN-λ1 treatments (Table 1). Among the three susceptible cells, TE-11 cells showed increased G0/G1 phase after 24 h and decreased

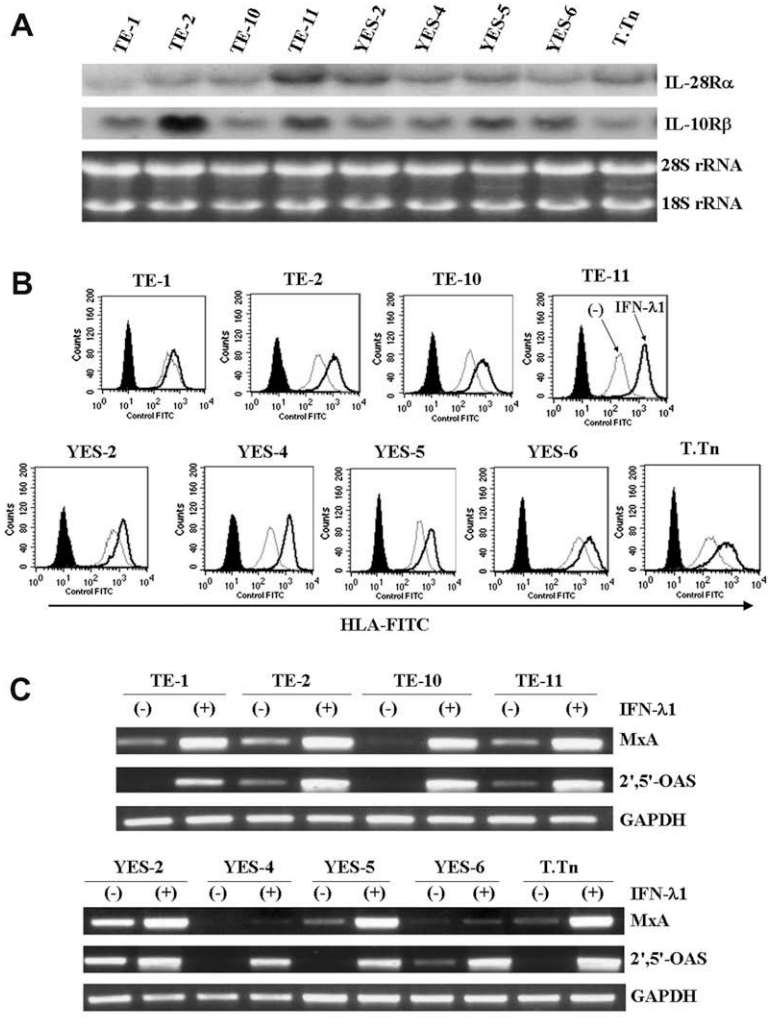

Fig. 1 – IFN-λ receptor expression and responsiveness to IFN-λ1 in oesophageal carcinoma cells. (A) Expression of the *IL-28Rα* and the *IL-10Rβ* genes was analysed with Northern blot analysis. Equal amounts of loading RNA were confirmed with PI staining of 28S and 18S rRNA. (B) Flow cytometrical analysis of MHC class I antigens expression. IFN-λ1-treated (black lines) or untreated (grey lines) cells were stained with anti-HLA Ab or isotopic-matched control Ab (black shaded). (C) Expression of the *MxA* and the *2′,5′-OAS* genes in IFN-λ1-treated and non-treated cells was tested with RT-PCR. Expression of the *GAPDH* gene was shown as an internal control.

G2/M subpopulations at 48 h, but YES-5 and T.Tn cells displayed different cell cycle patterns. Cell cycle synchronisation at G0/G1 phase with serum deprivation followed by serum plus IFN-λ1 treatment further clarified the effect of IFN-λ1 on cell cycle progression of TE-11 cells, showing that IFN-λ1 significantly prevented cell cycle exit from G1 phase arrest. Prolonged incubation with IFN-λ1 for 72 h did not increase sub-G1 populations in TE-11 cells (data not shown). We examined the involvement of cell cycle inhibitor p21 in the G1 phase arrest (Fig. 3). Expression of the *p21* gene was up-regulated in TE-11 cells but not in IFN-λ1 insensitive YES-6 cells (Fig. 3A). The expression was rather down-regulated in apoptosis-prone T.Tn cells probably because the apoptosis linked with decreased *p21* expression level.[20] Western blot analyses revealed that p21 but not p27 expression increased upon IFN-λ1 treatment and the p21 expression was down-regulated to an untreated level thereafter (Fig. 3B and C). Since phosphorylation of Rb permits the transition from G1 to S phase for cell proliferation, we examined the level of Rb phosphorylation in TE-11 cells and found that the phosphorylated level was continuously down-regulated after the IFN-λ1 treatment (Fig. 3B and C). These results demonstrated that IFN-λ1 induced G1 phase arrest in TE-11 cells with enhanced p21 expression and dephosphorylation of Rb.

3.4. *Apoptosis induction with IFN-λ1 treatment*

In contrast to TE-11 cells, cell cycle analyses showed increased sub-G1 fractions in YES-5 and T.Tn cells upon IFN-λ1 treatment (Table 1), suggesting that IFN-λ could induce apoptosis. The increased sub-G1 fractions became evident as early as 48 h (data not shown) after the treatment and continuously increased thereafter. In insensitive YES-6 cells, any significant changes of cell cycle were not observed upon

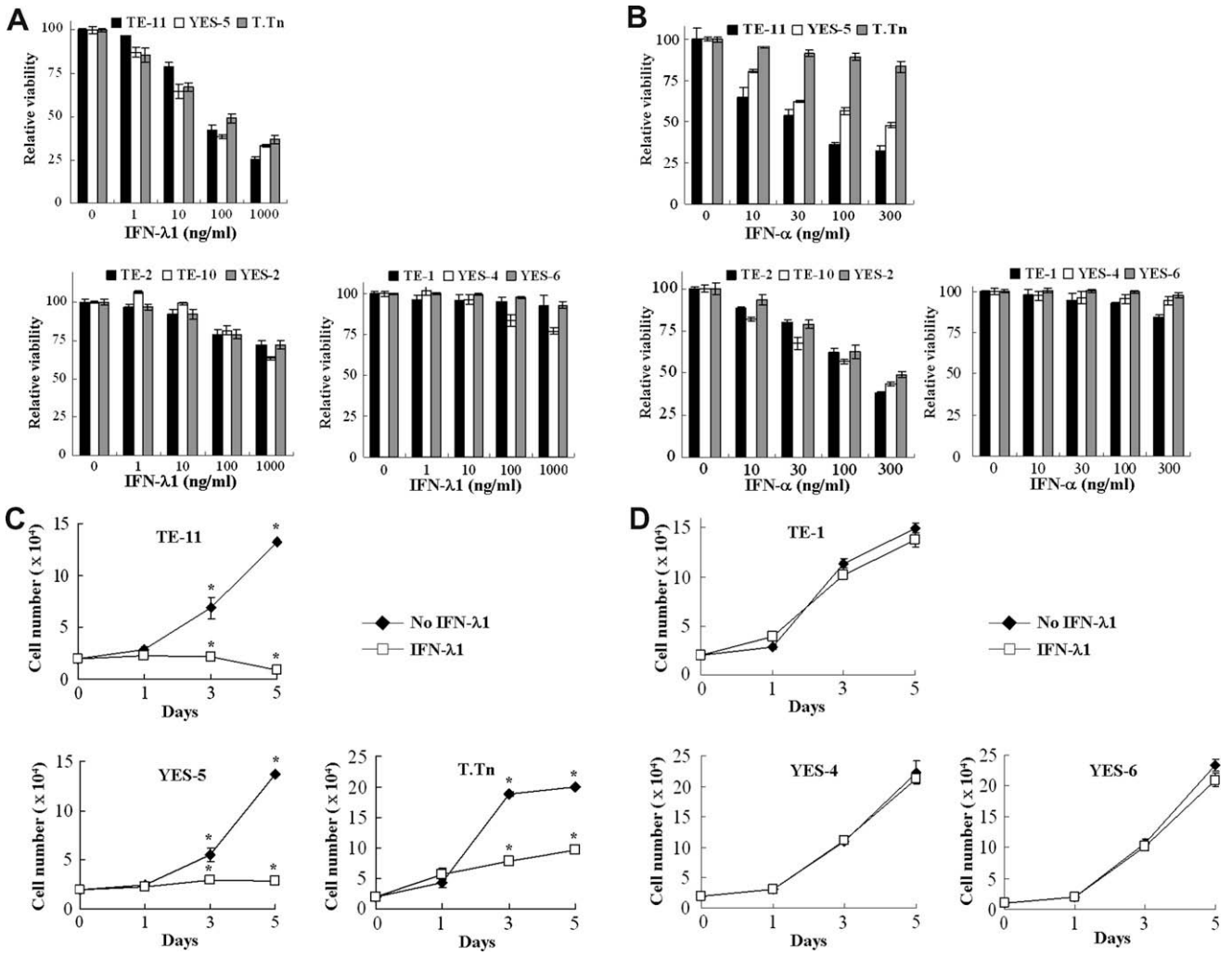

Fig. 2 – IFN-λ1- and IFN-α-mediated growth suppression in oesophageal carcinoma cells. Relative viability of cells treated with various amounts of IFN-λ1 (A) and IFN-α (B) was examined with the WST kit. Cells were divided into three groups regarding the responsiveness to IFN-λ1. (C, D) Cells were cultured with or without IFN-λ1 (100 ng/ml) and live cells numbers were determined with a dye exclusion test. Standard error bars are shown, *P < 0.01 (IFN-λ1 treated versus untreated).

IFN-λ1 treatments. We then examined flow cytometrical profiles with Annexin V and PI staining in YES-5 and T.Tn cells and demonstrated that the Annexin V^+/PI^- fraction and subsequently the Annexin V^+/PI^+ fraction increased with the treatment (Fig. 4A). We confirmed the activated apoptotic pathways by detecting cleaved caspase-3 and PARP in IFN-λ1-induced cells (Fig. 4B), and the caspase-3 cleavage was inhibited by a pan-caspase inhibitor Z-VAD-FMK but not a control Z-FA-FMK (Fig. 4C). These data collectively indicated that IFN-λ1 induced apoptosis in YES-5 and T.Tn cells.

3.5. *Restricted expression of IFN-λ receptors*

We investigated the IFN-λs receptor expression in other human tumour and normal cells (Fig. 5A). The expression of the *IL-10β* gene was ubiquitously detected in all the mesothelioma and pancreatic carcinoma cells tested, whereas the *IL-28Rα* gene was weakly expressed only in a mesothelioma cell line, NCI-H2052, and not expressed in any of the pancreatic carcinoma cells. In contrast to the IFN-λ receptors, all the tumour cells tested in the present study were positive for the IFN-α/β receptor complexes, *IFNAR1* and *IFNAR2* (Fig. 5A). We also examined the expression of the IFN-λs and IFN-α/β receptors in human normal cells. OUMS-24 and HFF fibroblast cells, and MSC expressed the *IFNAR1* and *IFNAR2* genes but none of them expressed the *IL-28Rα* gene. Interestingly, human non-transformed oesophageal epithelial Het-1A cells expressed both the IFN-λs and IFN- α/β receptors, suggesting that IFN-λs receptors expression is tissue-specific.

We confirmed that the receptor-negative normal and non-transformed cells were unresponsive to IFN-λ1. Treatment with IFN-λ1 did not increase the expression of the MHC class I molecules in OUMS-24, HFF or MSC cells, whereas IFN-α treatment up-regulated it (Fig. 5B). The expression level in Het-1A cells increased with both IFN-λ1 and IFN-α treatments. Likewise, induction of the *MxA* and the *2',5'-OAS* genes was not observed with IFN-λ1 treatment except in Het-1A cells, whereas IFN-α induced the expression in all the cells (Fig. 5C). We also confirmed that IFN-λ1 did not inhibit growth of all the cells including Het-1A cells but IFN-α

Table 1 – Cell cycle distribution after IFN-λ1 treatment.

Cell	Treatment	Time (h)	Cell cycle distribution (%)			
			Sub-G1	G0/G1	S	G2/M
TE-11	(–)	24	2.6 ± 0.3	42.4 ± 1.1	15.2 ± 0.3	40.4 ± 1.6
	IFN-λ1	24	4.0 ± 1.3	49.7 ± 1.2*	16.1 ± 1.4	40.8 ± 0.8
	(–)	48	3.7 ± 0.1	41.3 ± 0.3	18.9 ± 0.2	36.8 ± 0.3
	IFN-λ1	48	4.6 ± 0.2	53.3 ± 1.1*	18.4 ± 0.3	24.6 ± 1.4
Synchronised TE-11[a]		0	N.D.[b]	57.8 ± 0.5	18.0 ± 0.3	24.3 ± 0.4
	(–)	24	N.D.	30.3 ± 0.6	18.3 ± 0.6	51.2 ± 0.1
	IFN-λ1	24	N.D.	40.7 ± 0.8*	26.2 ± 0.7	33.1 ± 0.3
YES-5	(–)	72	9.3 ± 0.5	49.2 ± 1.3	20.7 ± 0.2	21.3 ± 0.6
	IFN-λ1	72	17.6 ± 1.9**	41.0 ± 1.0	20.9 ± 1.4	21.0 ± 0.5
T.Tn	(–)	72	2.5 ± 0.5	70.8 ± 0.1	11.7 ± 0.1	15.2 ± 0.3
	IFN-λ1	72	14.0 ± 1.1**	47.3 ± 0.7	21.3 ± 1.0	17.8 ± 0.9
YES-6	(–)	24	0.7 ± 0.1	52.4 ± 0.5	15.9 ± 0.2	31.6 ± 0.7
	IFN-λ1	24	0.7 ± 0.2	51.2 ± 0.4	16.3 ± 1.0	32.2 ± 0.5
	(–)	48	0.7 ± 0.2	48.6 ± 0.2	16.8 ± 0.4	34.3 ± 0.1
	IFN-λ1	48	1.3 ± 0.4	49.8 ± 1.0	17.6 ± 0.7	31.6 ± 0.5
	(–)	72	1.4 ± 0.2	56.6 ± 0.7	14.2 ± 1.0	28.2 ± 0.7
	IFN-λ1	72	1.8 ± 0.4	54.0 ± 0.5	16.1 ± 0.2	28.6 ± 1.1

Cells were treated with or without IFN-λ1 (100 ng/ml) for the indicated time. Cell cycle profiles were analysed with flow cytometry and the percent mean and the SE of each fraction are shown ($n = 3$).

* $P < 0.01$, comparing IFN-λ1-treated and non-treated cells at G0/G1 phase.

** $P < 0.01$, comparing IFN-λ1-treated and non-treated cells at sub-G1 phase.

[a] Cell cycle was synchronised at G0/G1 phase with serum deprivation and then released with serum only or serum containing IFN-λ1 (100 ng/ml).

[b] N.D., not determined since the values were quite small.

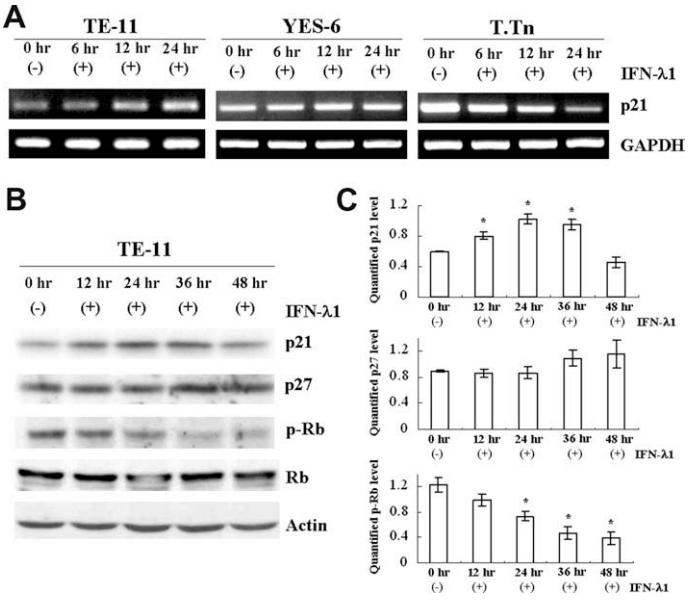

Fig. 3 – Induction of G1 phase arrest after IFN-λ1 treatment. Cells were incubated with IFN-λ1 (100 ng/ml) for the indicated periods. (A) Expression of the *p21* gene in TE-11, YES-6 and T.Tn cells was tested with RT-PCR. The *GAPDH* expression was also shown as an internal control. (B) A representative Western blot analysis on the expression of p21, p27, phosphorylated Rb at Ser795 (p-Rb) and total Rb (Rb) in TE-11 cells. Actin expression is shown as a loading control. (C) Quantified expression levels of p21, p27 and phosphorylated Rb as shown in (B) were determined by the relative intensity based on the corresponding actin (for p21 and p27) and total Rb (for phosphorylated Rb) expression. Intensity values of total Rb were further adjusted by the corresponding actin intensities. Average intensities with SE were calculated with three independent experiments. *$P < 0.05$ (IFN-λ1 treated versus untreated).

suppressed their growth in a dose-dependent manner ($P < 0.05$, above 30 ng/ml) (Fig. 5D). We also confirmed the unresponsiveness of Het-1A cells to IFN-λ1 with the growth kinetics (data not shown). Het-1A cells were thus insensitive

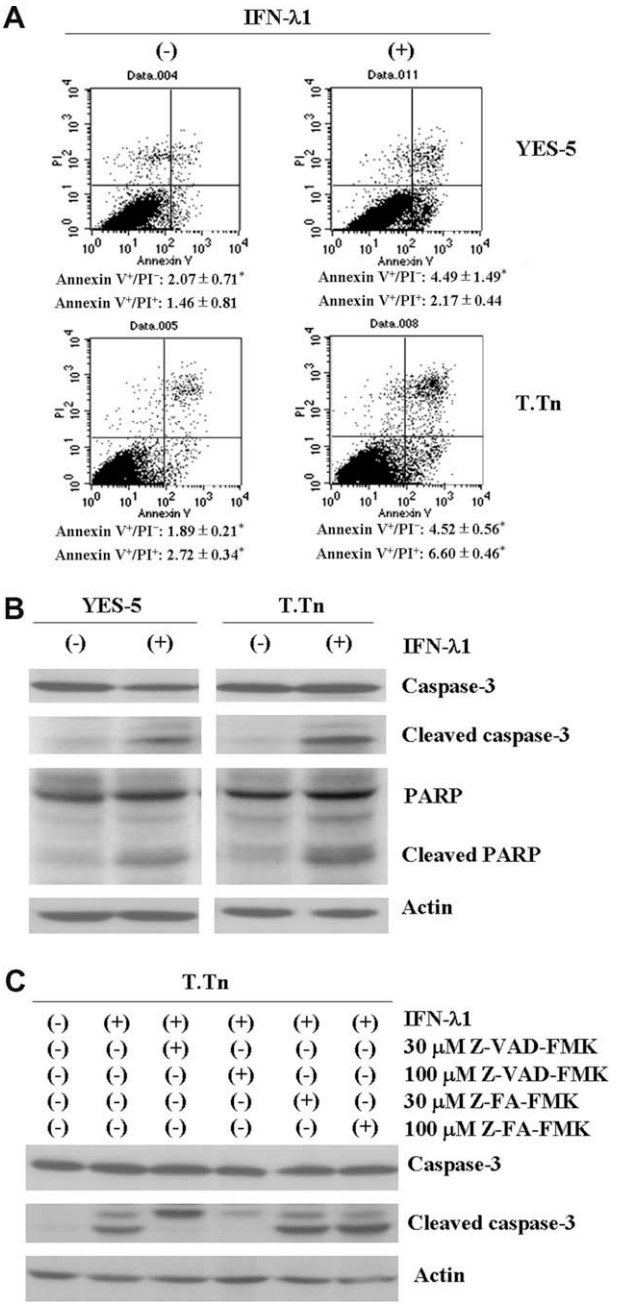

Fig. 4 – Apoptosis induction with IFN-λ1 treatment. (A) Cells treated with IFN-λ1 were stained with Annexin V and PI and representative profiles of flow cytometry are shown. The percentages of cell fraction with SE were calculated based on three independent experiments. *P < 0.05 (IFN-λ1 treated versus untreated). (B) Expression of caspase-3, PARP and the respective cleaved forms was analysed with Western blot analyses. Actin expression is shown as a loading control. (C) Cells were treated with Z-VAD-FMK or Z-FA-FMK as a control and then incubated with IFN-λ1. Expression of caspase-3, cleaved caspase-3 and actin as a control is shown.

to IFN-λ1-mediated growth inhibition although they showed up-regulation of the class I expression and induction of the anti-viral genes. These data demonstrated that Het-1A cells behaved like IFN-λ1-insensitive oesophageal carcinoma cells.

3.6. *Combination of IFN-λ1 and anti-cancer agents*

We investigated possible combinatory anti-tumour effects produced by IFN-λ1 and representative anti-cancer agents for oesophageal carcinoma, 5-FU and CDDP (Fig. 6A). IFN-λ1-sensitive TE-11, YES-5 and T.Tn cells were cultured with various doses of 5-FU or CDDP. We found that IFN-λ1 enhanced the cytotoxicity of 5-FU (IFN-λ1 30 ng/ml, TE-11 and T.Tn, $P < 0.01$ at 1 μM; YES-5, $P < 0.01$ at 0.1 μM) or CDDP (IFN-λ1 30 ng/ml, $P < 0.01$ at 1 μM) in a dose-dependent manner. The combinatory effects were additive irrespective of the agents. In contrast, IFN-λ1 scarcely influenced the cytotoxicity with 5-FU (IFN-λ1 300 ng/ml, $P > 0.26$ at 0.1 μM) or CDDP (IFN-λ1 300 ng/ml, $P > 0.07$ at 1 μM) in normal and Het-1A cells (Fig. 6B). IFN-α however enhanced the sensitivity to 5-FU (IFN-α 300 ng/ml, $P < 0.01$ at 0.1 μM) and CDDP (IFN-α 300 ng/ml, $P < 0.05$ at 1 μM) in normal and Het-1A cells (Fig. 6C).

4. Discussion

In this study we demonstrated an anti-proliferative activity of IFN-λ1 to oesophageal carcinoma by inducing G1 phase arrest or apoptosis and suggested a possible combinatory use for cancer therapy with anti-cancer agents. IFNs have a number of biological functions including anti-viral and immunomodulatory actions, which have also been demonstrated in type III IFN.[1,9,11,16] In contrast, the growth inhibitory actions of type III IFN have not been well investigated as well as the mechanisms although several studies reported that IFN-λ could suppress the cell growth of several tumour cell lines and intestinal epithelial cells.[5,12,13] We therefore examined the suppressive activity with panels of human tumours and normal cells, and found that the repertoire of IL-28Rα expression was tissue-specific and that the suppressive activity was subjected to cell types even originated from the same lineage. Although IFN-α and IFN-λ seem to share similar intracellular pathways[16], direct comparison of the inhibitory activity between the two IFN types with a number of cells has not yet been reported. The present data showed that IFN-λ1 was less effective than IFN-α in the suppressive activity to oesophageal carcinoma cells when tested at the same amounts. The differential suppressive ability however can be attributable to the distinct binding affinity of both IFNs to the respective receptors.

In terms of IFN-λ1-mediated inhibition, human oesophageal carcinoma cells were divided into 3 categories: sensitive, intermediate and insensitive. The cell type difference in growth inhibition was also found in IFN-α.[21] Moreover, variations of the IFN-λ1 sensitivity among cells were similar but not identical to those of IFN-α sensitivity, suggesting that both IFNs use comparable but discrete downstream pathways after the receptor ligation. Cell cycle analyses of the IFN-λ1-sensitive cells further demonstrated that IFN-λ1 induced either G1 phase arrest or apoptosis. IFN-λs was previously shown to inhibit cell proliferation through caspase-3-mediated apoptotic pathways.[12] The present data however firstly

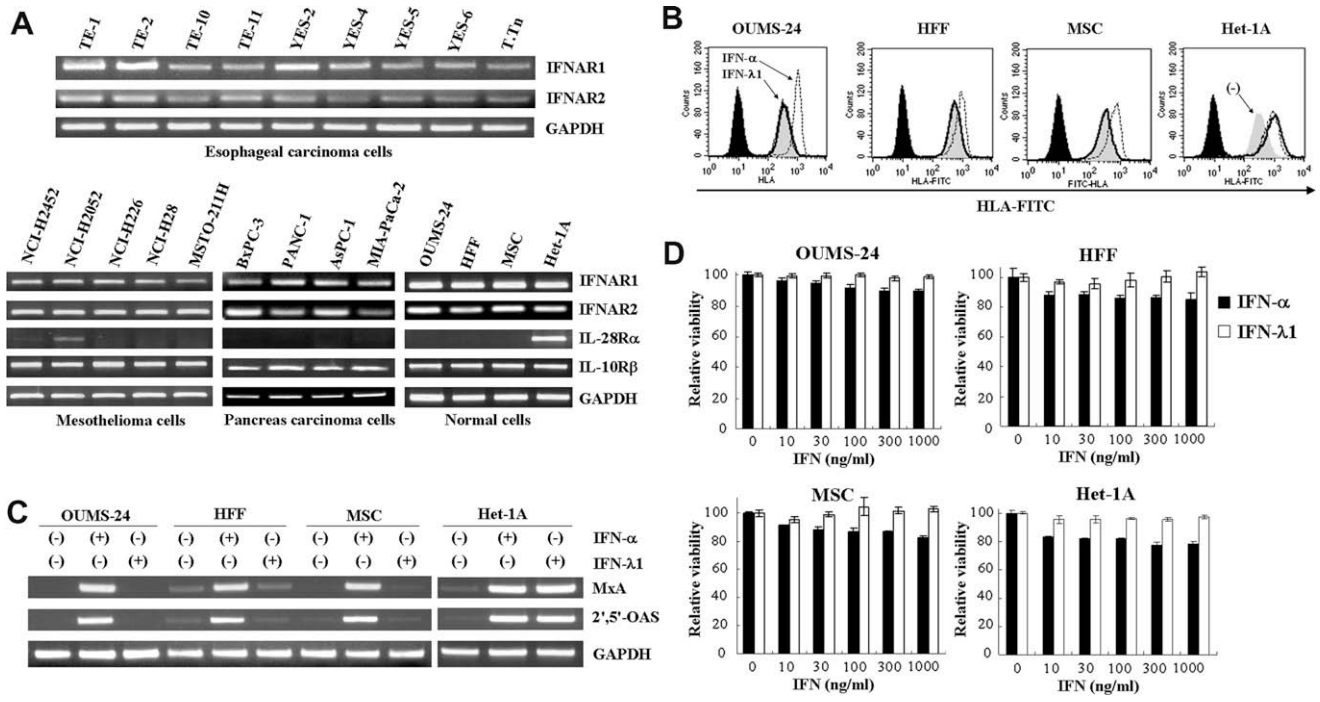

Fig. 5 – Responsiveness to IFN-λ and IFN-α in tumour and normal cells (A) Expression of the *IFNAR1*, *IFNAR2*, *IL-28Rα* and *IL-10Rβ* genes in mesothelioma, pancreatic carcinoma and normal cells was analysed with RT-PCR. Expression of the *IFNR1* and the *IFNR2* genes was also tested in oesophageal carcinoma cells as well. GAPDH is a control for cDNA amounts. (B) Flow cytometrial analysis of MHC class I antigens expression in normal cells. IFN-λ1 treated (black lines), IFN-α treated (dotted lines) or untreated (grey shaded) cells were stained with anti-HLA Ab or isotopic-matched control Ab (black shaded). (C) Expression of the *MxA* and *2′,5′-OAS* genes in IFN-α- or IFN-λ1-treated normal cells was analysed with RT-PCR. GAPDH is a control for cDNA amounts. (D) Viability of cells treated with various amounts of IFN-λ1 or IFN-α was tested with the WST kit. Standard error bars are shown.

demonstrated that IFN-λ1 also induced G1 phase arrest with p21 induction and Rb dephosphorylation and consequently revealed that IFN-λ1 achieved anti-proliferative activity through two different mechanisms, G1 phase arrest and apoptosis, depending on cell types. We also noticed that prolonged treatment of TE-11 cells with IFN-λ1 did not increase sub-G1 populations and that G1 phase arrest were not observed in YES-5 or T.Tn cells before their apoptosis induction (data not shown). These data suggest that the outcome of IFN-λ1 effects is distinctive with either G1 phase arrest or apoptosis. We also noticed a significant decrease of the *p21* transcript in IFN-λ1 treated-T.Tn cells, suggesting that the apoptosis can be linked with the down-regulated p21. Previous studies in fact showed that p21 up-regulation was linked with cell cycle arrest at G1 phase but rather inhibitory to apoptosis[22] and consequently the reduction of p21 was favourable to apoptosis initiation.[20] Down-regulated p21 expression could be a novel event related with the IFN-λ1-induced apoptosis.

In contrast to the differential susceptibility among the cells, up-regulation of MHC class I molecules and induction of anti-viral molecules were demonstrated in all the oesophageal carcinoma cells tested. The cell type difference detected in the growth inhibitory actions is therefore attributable to cell type-specific diversification in signal transduction pathways, which are distinct from those mediating MHC class I up-regulation and production of anti-viral molecules. It could also be due to additional escape mechanisms pertinent to the anti-proliferative effects in IFNs-insensitive cells.[23] The mechanisms of the resistance to IFN-λs may be heterogeneous as shown in IFN-α, which include a defect in the signal pathways of STATs[24] and an up-regulated expression of epidermal growth factor receptor resulting in amplified survival signals.[25] Since this study is the first report to our knowledge that the biological function of IFN-λ1 has been examined in a number of cells of the same origin, the differential responsiveness to IFN-λ1 among the oesophageal carcinoma cells could be a clue to investigate divergent signal pathways of IFN-λs. Marcello et al. also reported that IFN-λ activated IFN-stimulated gene repertoires with different manners from IFN-α in hepatitis C virus infection.[7] Similar investigations on IFNs-induced gene expression are required in the ligand-mediated growth suppression.

Type I IFNs enhance p53 expression and favour for apoptosis induction in the cells with intact p53 pathways.[26,27] It is currently unknown whether IFN-λs also increase p53 expression but the present study showed that the *p53* status of the oesophageal carcinoma cells was not directly correlated with the IFN-λ1-mediated anti-proliferative activity. The *p53* status of IFN-λ1-insensitive TE-2 and YES-6 cells was wild-type, whereas that of another insensitive YES-4 cells was mutated. IFN-λ1 sensitive TE-11 cells with the wild-type *p53* gene transiently up-regulated p21 expression but IFN-λ1 treatments had no effect on p53 protein levels and on the

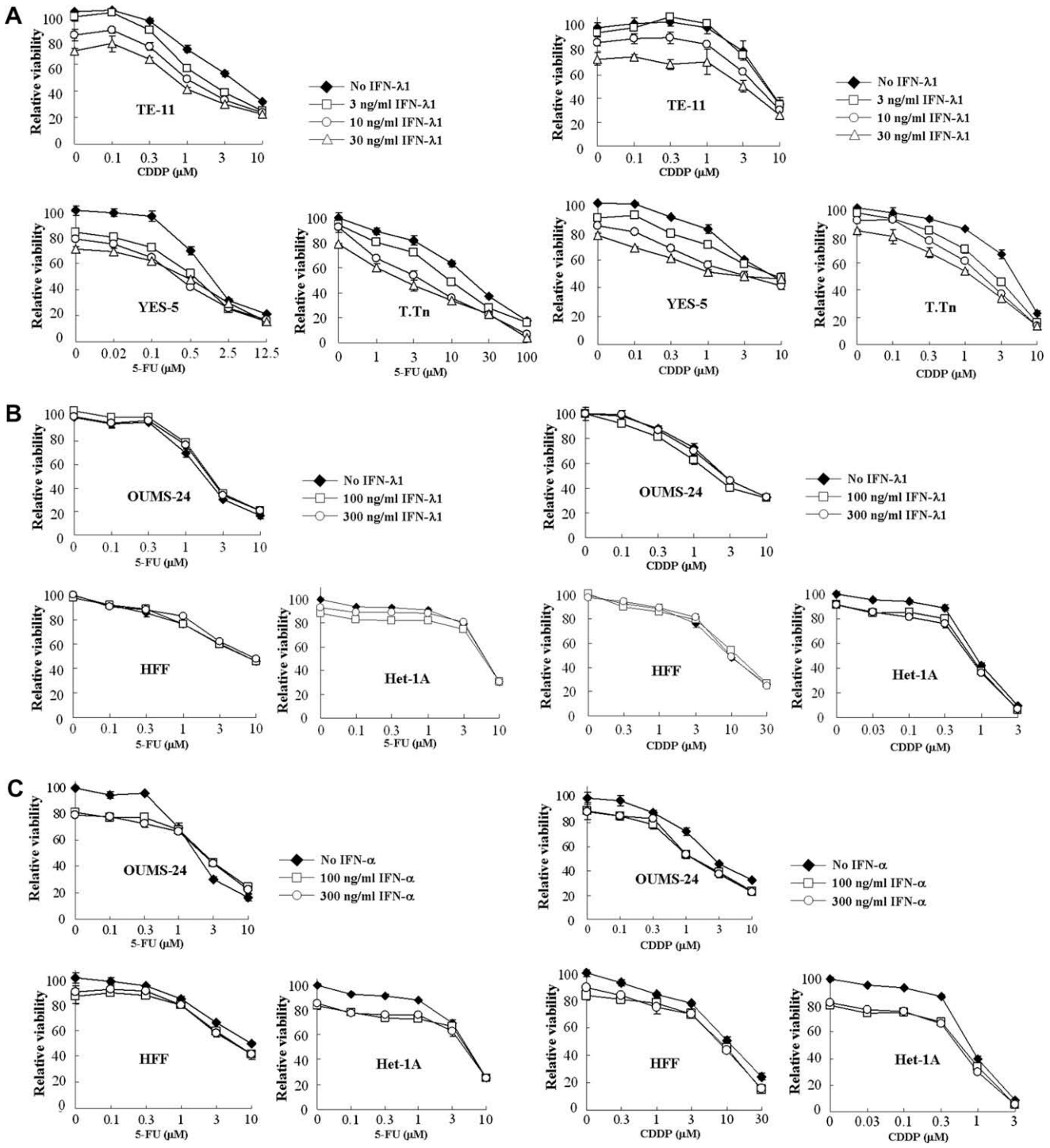

Fig. 6 – Combinatory effects of IFN-λ1 or IFN-α with anti-cancer agents to oesophageal carcinoma cells (A) and normal cells (B and C). Cells were cultured with IFN-λ1 (A and B) or IFN-α (C) together with either 5-FU (left) or CDDP (right) and viability of the cells was tested with the WST kit. Standard error bars are shown.

phosphorylation levels at serine 15 residue (data not shown), suggesting that the p21 up-regulation is p53-independent. The other sensitive YES5 and T.Tn cells were *p53*-mutated with an amino acid change but these cells were subjected to apoptosis, which probably is independent of the p53 functions. The p53-independent apoptosis by IFN-λ1 is firstly suggested in this study and it could be associated with death receptor-mediated apoptosis such as enhanced Fas expression, which was previously demonstrated with IFN-α treatemnts.[4,23] IFN-α can induce G1 phase arrest by up-regulating p21 with[28,29] or without p27,[30] but the involvement of p21 but not p27 in IFN-λ-mediated actions is also firstly demonstrated to our knowledge in the present study. The present data showed that IFN-λ1-induced-caspase-3 activation was

inhibited by Z-VAD-FMK; however a recent publication demonstrated that IFN-λ-induced apoptosis was not inhibited by Z-VAD-FMK,[31] suggesting caspase-independent apoptosis by IFN-λ as well.

One of the differential attributes between type I and type III IFN is a restricted receptor expression of IL-28Rα in contrast to ubiquitous expression of the type I IFN receptors. We showed that all the tumour and normal cells tested expressed both the *IFNAR1* and the *IFNAR2* genes; however, the *IL-28Rα* gene was expressed in the oesophageal carcinoma cells and the epithelium-derived Het-1A cells but not in other cells except NCI-H2052 cells expressing it at a low level. The *IL-10Rβ* gene was ubiquitously expressed as reported.[15] These data showed that oesophagus-derived cells were positive for IFN-λs receptor complexes, suggesting tissue-specific activation of IFN-λs-mediated pathways. In fact all the oesophageal carcinoma and the epithelial cells up-regulated in MHC class I molecules and produced anti-viral molecules upon IFN-λ1 treatment, whereas the receptors-negative normal cells did not. The normal cells however enhanced the class I expression and induced anti-viral molecules with IFN-α treatment. The initial report on IFN-λs demonstrated ubiquitous IL-28Rα expression in human,[2] but subsequent studies suggest that type III IFN actions could be specific to a certain cell subset including epithelial cells and several hematopoietic cell lineages.[5,32] The present data that mesothelioma and fibroblasts of epithelial origins were negative for the IFN-λ receptors suggest that the receptor distribution is more complex than that previously reported.[5,32] A phylogenic study showed that IFN-λs were precedent to IFN-α/β in the evolution.[33] The older type of IFNs therefore has rather a restricted target range. Biological significance of the narrow response window in contrast to broad type I IFNs spectrum remains unknown, which however suggests possible distinctive attributes of type III IFNs.

Limited expression of type III IFN receptors seems to be advantageous to cancer therapy. The present data showed that the growth of oesophageal carcinoma cells but not of normal cells was suppressed with IFN-λ1 in combination with anti-cancer agents. Since Het-1A is the only non-transformed cells available as originated from oesophagus and positive for the *IL-28Rα* gene, we cannot rule out the possibility that normal oesophageal epithelia in general could be sensitive to IFN-λ1. Nevertheless, in contrast to type I IFN, type III IFN may be less offensive for clinical use since systemic toxicities can be circumvented. Supposing that the potential activity to suppress tumour growth is the same between type I and type III IFNs, the latter is beneficial with the restricted target window. The present data in fact suggest that IFN-α is more potent in the growth inhibition than IFN-λ1 in the majority of oesophageal carcinoma cells; however, a recent report showed contradictory data that IFN-λ was stronger in the anti-proliferative activity than IFN-α.[34] Even in the present study we noticed that T.Tn cells were more susceptible to IFN-λ1 than IFN-α, suggesting that the differential sensitivity to respective IFNs depends on cell type. Currently IFN-α has been clinically in use for several malignancies and clinical trials showed that a combination of IFN-α and anti-cancer reagents such as 5-FU or CDDP had therapeutic effects on oesophageal carcinoma.[35] The major adverse effect of IFN-α is bone marrow suppression; however, IL-28Rα is not expressed in leukocytes.[15] We thereby presume that type III IFNs are less likely to cause the myelosuppression and can be tolerable even at high dose.

In conclusion, we demonstrated that IFN-λ1 suppressed proliferation of human oesophageal carcinoma cells by inducing G1 phase arrest or apoptosis with cell type specificity although it invariably up-regulated MHC class I expression and produced anti-viral molecules. The diversified mechanisms with respect to the cell type differences and the functional multiplicity need further investigations but the restricted expression of type III IFN receptor complexes, in sharp contrast to ubiquitously expressed type I IFNs receptors, characterises the limited biological effects. From the standpoint of a possible clinical application, IFN-λ1 could be a new chemo-sensitisation agent and a combinatory use with an anti-cancer agent can be a therapeutic strategy for a certain type of oesophageal carcinoma.

Conflict of interest statement

None declared.

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