IGFBP-6 plays a role as an oncosuppressor gene in NPC pathogenesis through regulating EGR-1 expression

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Abstract

Nasopharyngeal carcinoma (NPC) is prevalent in south-eastern Asia, particularly southern China, Singapore and Taiwan. The aim of this study was to identify the pivotal genes that may be altered during NPC progression. Using cDNA microarray analysis, we compared the expression of 18 genes between NPC and normal nasomucosal cells. qRT–PCR analysis found the expression of IGFBP-6 in NPC cell lines and immunolocalization of IGFBP-6 in NPC to be very weak. To explore the effects of IGFBP-6 on NPC tumour growth, we constructed inducible plasmids containing full-length IGFBP-6 cDNA (pBIG2i-IGFBP-6) and established pBIG2i-IGFBP-6-transfected NPC stable cell lines (NPC-TW01-pBIG2i-IGFBP-6). We then performed functional analysis of the IGFBP-6 in cell lines in vitro and in vivo. Over-expression of IGFBP-6 significantly suppressed the proliferation, invasion and metastatic activity of NPC cells and increased their apoptosis. We found the EGR-1, caspase-1 and TSP-1 genes to be markedly up-regulated when NPC-pBIG2i-IGFBP-6 was treated with doxycycline. Knocking down EGR-1 with EGR-1 siRNA resulted in a decrease in expression of caspase-1, TSP-1 and EGR-1 but not the expression of IGFBP-6. However, in knockdown cells the unchanged expression of IGFBP-6 did not inhibit the migration of NPC cells. Chromatin immunoprecipitation and luciferase reporter assay experiments showed that IGFBP-6 bound the EGR-1 promoter regions and activated EGR-1 promoter. We conclude that IGFBP-6 can regulate the progression of NPC by regulating the expression of EGR-1. These results suggest that IGFBP-6 could be used as a new target in NPC therapy.

Keywords: nasopharyngeal carcinoma (NPC); tumour progression; IGFBP-6; EGR-1

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Introduction

Nasopharyngeal carcinoma (NPC) is defined as a malignant tumour of the squamous metaplastic epithelium lining the surface and crypts of the nasopharynx [1]. It is a common malignancy among Chinese living in southern China, Taiwan and Singapore [2]. In Taiwan, the incidence of NPC is 5.3 cases/100 000 [3]. This special geographic and ethnic distribution of NPC may be related to hereditary, environmental or other factors. Although it has been suspected that certain hereditary factors, including expression of HLA-A2 and HLA-B46, are related the development of NPC, no major gene has been associated with the tumorigenesis of NPC [4]. Dietary factors such as eating salted fish are thought to increase the risk of NPC in Hong Kong and in low-risk populations [5]. Other environmental factors, such as long-term exposure to sulphuric acid vapour have also been suspected [6]. Some studies suggest a person to NPC [7–9] and others have suggested that EBV just enhances its development [10–12]. What we do know is that up-regulation of NF-κB2 and survivin is crucial in the tumourigenesis of NPC [13], and that in NPC cells the Wnt signaling pathway and RAS association domain family protein 1A (RASSF1A) pathway are also abnormally regulated [14,15]. However, our knowledge about the regulatory pathways of the genes involved in NPC tumourigenesis remains limited.

Previously, we established 10 NPC cell lines (NPC-TW01 to NPC-TW10) originating from people of Han Chinese origin living in Taiwan [16,17]. We used cDNA-microarray analysis to compare the expression of genes in these cell lines with that of normal nasomucosal epithelia (NNM) [18] and verified those findings...
Expression of IGFBP-6 in normal and cancer cells. (A) The qRT–PCR analysis of IGFBP-6 expression in normal nasal mucosa (NNM), polyp (NNP), NPC and other cancer cell lines. The normal nasal mucosa and polyps had higher expressions of IGFBP-6 mRNA, while the NPC and other cancer cell lines had rather weak expressions: CNE1, CNE2, BM-1 and Hone-1, nasopharyngeal carcinoma cell lines; CA127 and SAS, oral cancer line; Ca9-22, gingival carcinoma line; CaSk, epithelioid carcinoma line from the cervix; Hep3B, hepatocellular carcinoma line; PC3, prostatic carcinoma line. (B) The immunohistochemical staining shows almost no expression of IGFBP-6 in NPC-TW01 cell line (a) and NPC tumour nest in biopsy specimens (c). In normal nasal mucosa [arrows in (d)], IGFBP-6 was clearly expressed using quantitative RT–PCR (qRT–PCR) and immunohistochemistry. The expression of IGFBP-6 was found to be significantly lower in NPC and other cancer cell lines than in normal cells (Figure 1 see also Supporting Information, Table S1). IGFBP-6 has also been reported to be decreased more in human prostate epithelial cells as they become more tumourigenic [19]. Other studies reveal that the mRNA level of IGFBP-6 is lower in hormonally active carcinoma and pheochromocytoma than in normal adrenals [20], and transfection of IGFBP-6-expressing vectors into non-small cell lung cancer and neuroblastoma cells inhibit their proliferation and activate their apoptosis [21,22]. Thus, IGFBP-6 is suspected of being a significant oncosuppressor gene, although the underlying mechanism has not been identified. IGFBP-6 belongs to the IGFBP family 1–7, which are structurally related high-affinity binding proteins modulating the activity of IGFs [23]. IGFBP-6 has the highest binding affinity for IGF-II and it binds IGF-I with 20–100-fold lower affinity [24,25]. It is also a relatively specific inhibitor of IGF-II actions [26]. In this study, we investigated IGFBP-6 down-regulation in NPC, the role of IGFBP-6 in the pathogenesis of NPC and the possible mechanism underlying its oncosuppressive effect of NPC.

Materials and methods

Cell lines, tissues and biopsy specimens

The NPC cell lines used, NPC-TW01 to NPC-TW10, were established and characterized in our laboratory [16,17]. They and the plasmid-transfected NPC cell lines, with or without doxycycline treatment, were all cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 5% fetal calf serum (FCS), antibiotics and L-glutamine and incubated in a 10% CO₂ incubator. Normal nasomucosal (NNM) epithelia were cultured in DMEM containing 15% FCS.

NNM cells were primary cultures of normal nasomucosal epithelial cells derived from nasal polyps after polypectomy and were obtained from Dr D. H. Yeh of the ENT Department at National Taiwan University Hospital (NTUH), Taipei, Taiwan. The cells were first cultured in conditioned medium, as described previously [16]. After the sixth passage, the culture medium was replaced with DMEM containing 20% FCS. NPC paraffin blocks were obtained from the archives of the Department of Pathology, NTUH. The use of human specimens in this research was approved by the Institutional Review Board of NTUH.

RNA isolation and quantitative reverse transcriptase polymerase chain reaction (qRT–PCR)

We isolated total RNA by NucleoSpin® RNA II kit (Macherey-Nagel, Germany) from NPC cell lines, IGFBP-6 transfectants with or without drug treatment and the control normal nasomucosal epithelia (NNM), according to the manufacturer’s directions.

Total RNA was then reverse-transcribed to cDNA using a SuperScript III RT Kit (Invitrogen, CA, USA) and qRT-PCR was analysed with an ABI prism 7700 Sequence Detector System and SYBR Green PCR
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Master Mix Kit (Perkin-Elmer, Applied Biosystem, Wellesly, MA, USA), according to the manufacturer’s directions. GAPDH was used as an endogenous reference gene.

Immunohistochemical localization of proteins in NPC cell lines and biopsy specimens

NPC paraffin blocks were sliced into 5 µm-thick tissue sections and collected on coated slides. After deparaffinization, the sections were stained with haematoxylin and eosin (H&E) and then immunostained according to previously described methods [11,12]. The same method was also used to localize IGFBP-6 and other antigens in SCID mice bearing NPC transfectants in metastatic tumour nodules in the other organs.

Promoter methylation evaluation

To verify whether the IGFBP-6 promoter had been hypermethylated endogenously, NPC cells were treated with 5-aza-2-deoxycytidine (Sigma, MO, USA) at a concentration of 2 µM. After 5 days, the RNA was extracted for qRT–PCR analysis of IGFBP-6 expression. The cells not treated with 5-Aza-dC were used as controls.

Establishment of the stable NPC cell line transfected by an inducible (tet-on) plasmid containing IGFBP-6-cDNA (pBIG2i-IGFBP-6)

The IGFBP-6 cDNA was obtained by reverse transcription of the total RNA from normal nasoamucosal epithelia. After PCR amplification, a fragment including the 983 bp IGFBP-6 open reading frame was obtained and cloned into the tet-on expression vector pBIG2i which activated transcription in the presence of doxycycline (Merck). This plasmid was designated as pBIG2i-IGFBP-6. The IGFBP-6 in this vector was shown by electrophoresis and PCR analysis. The pBIG2i that did not contain IGFBP-6 was used as a control. For transfection, lipofectamine (Invitrogen) was employed as previously described [27]. For establishment of stable NPC-TW01 transfectant cell line, the reader is referred to the Supplementary information, supplementary methods.

MTT [3-(4, 5-dimethylthiazolo-2yl)-2,5-diphenol tetrazolium bromide] assay

The stable-transfected NPC cells containing pBIG2i-IGFBP-6 were plated onto a 96-well plate at 2000 cells/well, separately. After doxycycline (2 and 4 µg/ml) had been added and they had been incubated 0, 1, 2 and 3 days, the cells underwent MTT assay, as described previously [28].

Tumour cell migration and invasion ability assay

For migration assay, a pipette tip was used to scratch a wound (about 2–3 mm in width) on a monolayer of NPC-TW01, NPC-TW01 IGFBP-6 transfectant with and without doxycycline treatment and NPC-TW01 IGFBP-6 and EGR-1 siRNA transfected treated with doxycycline. A membrane invasion chamber system (MICS) was used to evaluate the invasion ability of transfected NPC cells with expression of IGFBP-6. In the chamber, we used a nitrocellulose (NC) membrane with a pore size of 8 µm (Falcon Fluoroblock insert, 24 well format) coated with 10 mg/ml Matrigel (50 µl/well). The invasive cells that had migrated to the bottom side of the NC membrane were fixed with methanol and stained with Giemsa solution. The degree of migration and invasion was evaluated following previous protocols [27].

NPC-TW01-pBIG2i-IGFBP-6 tumour growth in SCID mice

6 × 10⁶ pBIG2i-IGFBP-6-transfected stable NPC cells were transplanted into 12 SCID mice subcutaneously. The animals were divided into two groups: one with 2 mg/ml doxycycline [29] and 2% sucrose added to their drinking water, and the other with 2% sucrose only added to their drinking water. The tumour size was measured once a week for 13 weeks. Their volumes (V) were calculated using the following equation:

\[ V = L \times W^2 \times 0.52 \]

where \( L \) is the length and \( W \) the width of the tumour mass. At 13 weeks, the animals were killed and subjected to routine autopsy. The tumour masses were dissected and prepared for paraffin sectioning and stained with H&E for morphological observation and also immunostained with anti-IGFBP-6 antibody.

Transfection of small interfering RNA (siRNA)

After screening several well-known oncosuppressor genes by qRT–PCR, we hypothesized that the well-known oncosuppressor gene EGR-1 [30,31] may play some role in IGFBP-6’s inhibition of tumour cell growth, migration and invasion. To verify the relationship between IGFBP-6 and EGR-1, we performed transient transfection of EGR-1 small interfering RNA (EGR-1 siRNA; Santa Cruz Biotechnology, Germany) to NPC-TW01 IGFBP-6 and EGR-1 siRNA transfectants treated with doxycycline, according to the manufacturer’s directions, as previously reported [27].

Western blotting of co-immunoprecipitation (Co-IP) of IGFBP-6 and EGR-1 proteins

For Co-IP that characterized the interaction between IGFBP-6 and EGR-1 proteins, the reader is referred to the Supporting information (Supplementary methods).

Chromatin immunoprecipitation (ChIP) analysis

The ChIP assay was carried out using a Chromatin Immunoprecipitation Assay kit (Upstate-Millipore, Wellsely, MA, USA), according to the manufacturer’s directions. GAPDH was used as an endogenous reference gene.
Based on the results of ChIP, the forward primer PCR product was then used as the second template. The forward and reverse primers were 5′-CTGGGTTGACACAGGGAGACT-3′ and 5′-CCACTGCTTACCTGCGGT-3′, respectively. The PCR product was then used as the second template. The PCR product to T vector and sequenced using the forward and reverse primers.

Luciferase reporter assay

The assay was performed as described on our previous studies [32]. Briefly, nest PCR was performed and the DNA of NPC-TW01 was used as the first template. The forward and reverse primers were 5′-GGACAGCCACAGGGATT-3′ and the reverse primer was the transcription start site (TSS), 5′-GCTGGGATCTCTCGCGA-3′. The PCR product was then used as the second vector. To obtain the fold enrichment value, we further normalized the IP : Input value to non-specific loading control GAPDH as reference. As for mock ChIP, we used normal mouse IgG as the control antibody. For the primers used for ChIP analysis, the reader is referred to the Supporting information (Supplementary methods).

Results

IGFBP-6 expression in NPC cell lines and biopsy specimens

To identify molecules with biological significance in NPC tumourigenesis, we selected 18 genes by cDNA microarray (see Supporting information, Table S1A). Each gene expression was verified by qRT–PCR analysis, using four sets of normal nasomucosal epithelia and 10 sets of NPC cell lines. We found IGFBP-6 to be very different in expression in NPC cells than in normal epithelial cells. Examining IGFBP-6 gene expression by qRT–PCR, we found the expression of IGFBP-6 to be an average of five-fold lower in NPC cell lines than in NNM cells (Figure 1A). To further confirm the down-regulation of IGFBP-6 in NPC cells, we used immunostaining to detect expression of IGFBP-6 in NPC biopsy specimens. Immunolocalization of IGFBP-6 in 15 NPC biopsy specimens showed that IGFBP-6 protein was expressed in the normal epithelial cells but not in the tumour nests (Figure 1B).

Since most NPC cell lines showed very weak expression of IGFBP-6, we wanted to know whether their promoters had been hypermethylated. After NPC cells had been treated with 2 μM 5-aza-2-deoxycytidine for 5 days, our qRT–PCR found no increase in the expression of IGFBP-6 mRNA. Compared with an ovarian endometrioid carcinoma (P4) cell line (our parallel control), there was a four-fold increase in expression of IGFBP-6 in the P4 cell line after treatment with 5-aza-2-deoxycytidine (see Supporting information, Figure S1). These findings suggest that the low expression of IGFBP-6 was not a result of hypermethylation of its promoter region.

Functional analysis of IGFBP-6 in NPC-TW01 and in SCID mice bearing NPC xenografts

To directly investigate whether IGFBP-6 could regulate tumourigenesis, we engineered ectopic expression through stable transduction of NPC-TW01 cells with a tetracycline-responsive IGFBP-6 expression vector (pBIG2i-IGFBP-6). After doxycycline induction, the pBIG2i-IGFBP-6-transfected NPC-TW-01 cells (NPC-TW01-pBIG2i-IGFBP-6) strongly expressed IGFBP-6 mRNA (Figure 2A) and protein (Figure 2B). The same results were found in our immunohistochemical studies, revealing an increase in production of IGFBP-6 protein in NPC-TW-01 after doxycycline induction (Figure 2C).

We found IGFBP-6 to be repressed in tumour cells (Figure 1), raising the possibility that re-expression of IGFBP-6 might inhibit tumour growth. To clarify the functional role of IGFBP-6, we used MTT to investigate the effect of IGFBP-6 on the growth of cancer cells. We found fewer cells in doxycycline-treated NPC-TW01-pBIG2i-IGFBP-6 than in the untreated cells, suggesting that IGFBP-6 could inhibit the proliferation ability in the NPC-TW01 cell line (Figure 3A).
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Figure 2. Over-expression of IGFBP-6 by doxycycline (Dox) induction in stable transfectant NPC-TW01 cell. (A) qRT–PCR (lane 1, NPC-TW01; lane 2, NPC-TW01 + Doxy; lane 3, NPC-TW01-pBIG2i-IGFBP-6; lane 4, NPC-TW01-pBIG2i-IGFBP-6 + Doxy). The Doxy-treated cells expressed >200-fold IGFBP-6 mRNA (lane 4) than the control cells. *p < 0.001. (B) The culture media from NPC lines were concentrated to 100-fold and subjected to western blot analysis. The Doxy-treated NPC-TW01-pBIG2i-IGFBP-6 cells expressed five-fold more IGFBP-6 than NPC-TW01 cells as measured by densitometry (lanes 1 and 6). (C) Immunohistochemistry also showed that IGFBP-6 could only be clearly detected in the NPC-TW01-pBIG2i-IGFBP-6 cells treated with Doxy.

In addition, 24 h treatment with doxycycline markedly suppressed the migration ability of NPC-TW01 cells with over-expressed IGFBP-6 (Figure 3B). Invasion ability was also markedly inhibited in the IGFBP-6-over-expressed NPC-TW01 cells (Figure 3C, D). These results show that IGFBP-6 inhibited tumour cell proliferation, migration and invasion ability.

We investigated the inhibition of IGFBP-6 in NPC tumour growth in vivo, using SCID mice bearing NPC xenografts derived from the NPC-TW01-pBIG2i-IGFBP-6 cell line. We found a greater reduction in tumour growth rate in the doxycycline-treated group than in the untreated mice bearing xenografts (Figure 4). By 13 weeks, the average weight and volume of doxycycline induction xenografts had decreased 75% and 64%, respectively, compared to the control group. Histopathological specimens from the doxycycline-treated NPC-TW01-pBIG2i-IGFBP-6 xenografts showed tumour shrinkage, increased apoptosis and necrosis in the whole tumour (Figure 4B1). There were no metastatic nodules in the visceral organs in the SCID mice treated with doxycycline (Figure 4B2, B3), while in untreated SCID mice there were many metastatic nodules on the visceral organs (Figure 4A2, A3). To rule out the possibility that the oncosuppressive phenomenon was specific to NPC-TW01, we repeated in vitro and in vivo studies of the NPC-TW02 line and found similar results (data not shown). These findings demonstrate that IGFBP-6 is oncosuppressive for NPC.

EGR-1 expression is associated with IGFBP-6-over-expressed NPC-TW01 cells

To study the downstream gene of IGFBP-6, we analysed several tumour suppressor genes and found an association between EGR-1 expression and doxycycline-treated NPC-TW-01-IGFBP-6. qRT–PCR analysis showed up-regulated expression of EGR-1 in NPC cells with over-expressed IGFBP-6 (Figure 5B, lane 2). Furthermore, double localization of IGFBP-6 and EGR-1 revealed that both proteins were partially co-localized in the same site at each tumour cell (Figure 5E). To further confirm the association between the expression of EGR-1 and IGFBP-6-over-expressed
NPC-TW01 cells, we checked whether or not EGR-1 was expressed in tumour xenografts of doxycycline-treated mice and found that it was expressed following the over-expression of IGFBP-6 (Figure 4). This result was similar to that of our immunofluorescent studies of doxycycline-treated NPC-TW-01-IGFBP-6 (Figure 5E). However, our co-immunoprecipitation experiment did not find a direct interaction between IGFBP-6 and EGR-1 protein (Figure 6A). Thus, EGR-1 may be a gene that is targeted by IGFBP-6 in its suppression of NPC-TW01 proliferation and its induction of necrosis and apoptosis.

The oncosuppressor regulation of IGFBP-6 occurs through regulating the expression of EGR-1

The transcription factor EGR-1 has recently been identified as a direct regulator of multiple tumour suppressors, such as TGFβ1, PTEN, p53 and fibronectin, and it has been proposed that it can be used as a target for gene therapy for prostate cancer [32]. We rechecked EGR-1 in NNM lysate and tumour xenograft and found that its expression paralleled IGFBP-6 (see Supporting information, Figure S3). To investigate the mechanism that underlies that ability of IGFBP-6 to suppress tumour activity via EGR-1, we treated NPC-TW01-pBIG2i-IGFBP-6 cells with EGR-1 siRNA. Once the EGR-1 siRNA had been transfected into doxycycline-treated NPC-TW01-pBIG2i-IGFBP-6 cells, there was significant suppression of EGR-1, Caspase-1 and TSP-1 (Figure 5B–D, lane 4) but no change in IGFBP-6 via qRT–PCR (Figure 5A, lane 4). Western blot analysis showed similar results (data not shown). These findings suggest that EGR-1 is the gene that IGFBP-6 targets downstream. Furthermore, inhibition of NPC cell migration via IGFBP-6 was reversed when the cells were treated with EGR-1 siRNA (Figure 5E). Together, these results indicate that IGFBP-6’s inhibition of the growth and migration of NPC is mostly dependant on EGR-1.

IGFBP-6 binding to EGR-1 promoter and up-regulating EGR-1 promoter activity

We further investigated the relationship between IGFBP-6 and EGR-1 by performing ChIP assays to confirm IGFBP-6 binding to regulatory sequences of EGR-1 promoter. As shown in Figure 6B, there was a three- and four-fold increase, respectively, in IGFBP-6 binding to EGR-1 promoter in IGFBP-6-expressed NPC cells. In contrast, in IGFBP-6-negative NPC cells,
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Figure 4. (A) The effects of IGFBP-6 over-expression on in vivo tumour xenograft growth of NPC–TW01. The tumour volume (A) and tumour weight (B) of NPC xenograft decreased significantly when IGFBP-6 was over-expressed 13 weeks later. *p < 0.05. (B) Histopathological morphology and immunostaining show in IGFBP-6 over-expressed xenograft revealing significant apoptosis and necrosis (B-1) compared to xenograft without IGFBP-6 over-expression (A-1). Kidney (A-2) and lung (A-3) in the untreated mice showed multiple metastatic nodules, while in the mice given drinking water with 2 mg/ml Doxy, no metastatic nodules could be detected (B-2, B-3). (C) The Doxy-treated NPC-TW01-pBIG2i-IGFBP-6 cells expressed plenty of IGFBP-6 (a) and EGR-1 (c), while NPC-TW01-pBIG2i-IGFBP-6 cells not treated with Doxy expressed almost no IGFBP-6 (b) or EGR-1 (d). No reaction product could be detected in Doxy-treated NPC-TW01-pBIG2i-IGFBP-6 cells when control antibody was used (e).

there was only background level binding of IGFBP-6 to EGR-1 promoter (Figure 6B). These findings suggest that by binding to EGR-1 promoter and increasing EGR-1 expression, IGFBP-6 can trigger the suppression of tumours. The up-regulation of the EGR-1 promoter activity by IGFBP-6 was further confirmed by luciferase reporter assay. The luciferase activity was increased 3.4-fold in pGL3-EGR1-Luc-transfected cells compared with pGL3-Luc-transfected cells (Figure 6C). The results of ChIP and luciferase reporter assays showed that IGFBP-6 bound to the promoter and up-regulated the promoter activity of EGR-1.

Discussion

The insulin-like growth factor binding protein (IGFBP) family, which consists of seven distinct gene products (IGFBPs 1–7) [23], has been shown to suppress tumours dependent on and independent of IGF [34–36], although the mechanism underlying their suppressive ability has remained poorly understood.

IGFBP-6 acts as the effector of tumour suppressor SEMA3B (class 3 semaphorins) in human lung cancer NCI-H1299 cells [36], and it activates apoptosis in non-small cell lung cancer [37] and inhibits cell proliferation in an androgen-independent prostate cancer cell line, PC-3 [38]. Over-expression of IGFBP-6 inhibits the proliferation and promotes the apoptosis of rhabdomyosarcoma in vitro and inhibits xenograft growth in vivo [39]. It also inhibits IGF-II-induced proliferation of colon cancer cells [26].

In this study, we found IGFBP-6 to be decreased in various NPC cell lines and the tumour cells in biopsy specimens but not in normal nasal mucosal cells. We detected IGFBP-6 immunoreactivity only in the normal nasal mucosa but not in the cancer cells themselves (Figure 1B). IGFBP-6 transcripts and proteins were found to have a significant reduction in NPC cell lines as well as cancer cells in
Figure 5. Effects of EGR-1 up-regulation in NPC-TW01. (A–D) In IGFBP-6 over-expression NPC-TW01, EGR-1, Caspase-1 and TSP-1 are all over-expressed (A–D, lane 2). But in EGR-1 knock-down NPC-TW01-pBIG2i-IGFBP-6 + Doxy, expression of EGR-1, Caspase-1 and TSP-1 decreased significantly (B–D, lane 4), *p < 0.05, while IGFBP-6 almost remained at the same level of expression (A, lane 4). (E) Immunofluorescent localization of IGFBP-6 and EGR-1. In NPC-TW01-pBIG2i-IGFBP-6 (A1, A2), expressions of both IGFBP-6 and EGR-1 proteins were very weak, while expressions of both proteins increased significantly in the Doxy-treated cells (B1, B2). Also, double-localization images showed partial co-localization in each cancer cell (B3). (F) IGFBP-6 lost its partial ability to inhibit NPC cell migration in EGR-1 knock-down NPC cells (panels 5, 6).

Because inhibiting methyltransferase did not increase the expression of IGFBP-6, we ruled out the possibility that its down-regulation was due to hypermethylation of its promoter (see Supporting information, Figure S1). When intracellular IGFBP-6 was over-expressed, there was marked inhibition of tumour cell proliferation rate, migration and invasion ability and a clear reduction of xenograft tumour growth of NPC-TW01 (Figures 3, 4). These findings are consistent with previous in vivo or in vitro studies of IGFBP-6 in other cancers [36–39].

To investigate the mechanisms of oncosuppressor function of IGFBP-6, we found that EGR-1 was up-regulated in NPC-TW01-pBIG2i-IGFBP-6 cells treated with doxycycline, using qRT–PCR, immunohistochemistry and immunofluorescence analyses (Figure 5). EGR-1 is a nuclear protein that contains three zinc fingers of the C2H2 subtype. EGR-1 is known as an oncosuppressor [31,40–42] and has been shown to suppress the growth and transformation of human fibrosarcoma cells by inducing transforming growth factor-β1 [43], up-regulating the antitumourgenic NSAID-activated gene 1 (NAG-1) by Sulindac Sulfide [44], cooperating with Sp1, Jun-B and p21WAF1/Cip1 and stimulating apoptosis by transactivating the p53 gene [45]. EGR-1-induced apoptosis has also been reported in p53−/− cells, suggesting the possible existence of both p53-dependent and p53-independent pathways [46]. EGR-1 is also a direct regulator of at least four major suppressors, TGFβ1, PTEN, p53 family members and fibronectin [33]. We found basal expression of EGR-1 in NPC, like IGFBP-6, to be very low in our study (Figure 5B, lane 1), as has been reported by previous studies of other tumour cells [46,47]. However, we found EGR-1 to be up-regulated simultaneously when IGFBP-6 was over-expressed (Figure 5B, lane 2). Because the IGFBP-6 expression level did not change significantly when EGR-1 was knocked down, EGR-1 could be the...
Figure 6. Interaction between IGFBP-6 and EGR-1. (A) Co-immunoprecipitation (Co-IP) of IGFBP-6 and EGR-1 in NPC-IGFBP-6 transfectant with or without Doxy treatment. (a) Western blotting (WB) with anti-EGR-1. Using anti-IGFBP-6 for IP, no EGR-1 protein was seen in lanes 1 and 3. When anti-EGR-1 was used for IP, significant immunocomplex protein was shown in lanes 2 and 4. (b) Western blotting (WB) with anti-IGFBP-6. When anti-IGFBP-6 was used for IP, a large immunocomplex protein was detected in lanes 1 and 3. However, no IGFBP-6 protein could be seen in lanes 2 or 4 when anti-EGR-1 was used for IP. (B) Quantitative ChIP analysis of IGFBP-6 binding to EGR1 promoter. (a) Schematic presentation of the IGFBP-6 binding sites in EGR-1 promoter from -1827 to -1719 bp and from -1237 to -1119 bp. TSS, transcription start site. (b) Significant enrichment of IGFBP-6 binding to EGR1 promoter was seen in NPC-TW01-pBIG2i-IGFBP6+Doxy cells, but not in NPC-TW01 cells, whose level of binding was similar to the controls with normal mouse IgG antibodies (*p < 0.05). (C) The regulation of EGR1 promoter activity by IGFBP-6 in NPC cells. The EGR1 promoter reporter (pGL3-EGR1-Luc) or control vector pGL3-Luc was transfected into NPC TW01-IGFBP6 cells for 48 h, and the EGR1 promoter expression was measured by luciferase assay. The luciferase activity was measured in each cell lysate and reported in arbitrary units. The same experiment was repeated three times. All data are reported as average values. **p < 0.01.

gene that IGFBP-6 targets downstream to execute the suppression of tumour growth. In this study, IGFBP-6 up-regulated caspase-1 and TSP-1 (Figure 5C, D, lane 2), while EGR-1 siRNA down-regulated caspase-1 and TSP-1 (Figure 5C, D, lane 4), indicating that both IGFBP-6 and EGR-1 target caspase-1 and TSP-1.

We used ChIP analysis and luciferase reporter assay to study the relationship between IGFBP-6 protein and the EGR-1 promoter region, and found that IGFBP-6 could bind to EGR-1 promoter regions and regulate the expression of EGR-1 (Figure 6B, C). Considered together, these experiments indicate that IGFRP-6 is an oncosuppressor gene that, by acting as a transcription factor, can regulate the expression of EGR-1 to suppress the NPC tumourigenesis. In conclusion, our experiments suggest that IGFBP-6 may play an important role in regulating the progression of NPC. IGFBP-6 is ubiquitously down-regulated in all NPC cell lines and various cancer cells. Since IGFBP-6 represses cell proliferation, migration invasion and tumour growth, it may emerge as an important target for NPC gene therapy. Further understanding of the biology of IGFBP-6 in cancer may yield new strategies for the diagnosis and treatment of cancer.

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Statement of author contributions

Yuan-Sung Kuo designed and carried out experiments and collected data, Yueh-Bih Tang conceived the experiments, Tung-Ying Lu carried out the experiments of ChIP and Luciferase reporter assay, Han-Chung Wu analysed and interpreted data and Chin-Targn Lin conceived the experiments, analysed and interpreted data. Yuan-Sung Kuo, Han-Chung Wu and Chin-Targn Lin were involved in writing the paper and all authors had final approval of the submitted and published versions.

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