Nuclear epidermal growth factor receptor interacts with transcriptional intermediary factor 2 to activate cyclin D1 gene expression triggered by the oncoprotein latent membrane protein 1

Yin Shi1,4, Yongguang Tao1,4,†, Yiqun Jiang1,4, Yang Xu1,5, Bin Yan1,4, Xue Chen1,4, Lanbo Xiao1,4, and Ya Cao1,4,†

1Cancer Research Institute and 2Molecular Imaging Center, Central South University, Changsha, Hunan 410078, China, 3Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Hunan 410078, China, 4Key Laboratory of Carcinogenesis, Ministry of Health, Hunan 410078, China and 5Department of Gastroenterology, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410005, China

*To whom correspondence should be addressed. Email: taoyong@mail.csu.edu.cn. Tel +86-731-84805448; Fax +86-731-84470589
Correspondence may also be addressed to Ya Cao. Email: ycao98@vip.sina.com. Tel +86-731-84805448; Fax +86-731-84470589

The epidermal growth factor receptor (EGFR), a ubiquitously expressed receptor tyrosine kinase, is an important factor in carcinogenesis. Transcriptional intermediary factor 2 (TIF2), a member of the p160 nuclear receptor co-activator gene family, is linked to the proliferation of cancer cells. However, the direct interplay between the EGFR and the nuclear receptors remains unclear. Our previous study demonstrated that nuclear EGFR could directly bind to the cyclin D1 promoter under the regulation of the oncoprotein latent membrane protein 1 (LMP1), but it also indicated that other factors are involved in the activation of target genes. In this study, we found that LMP1 upregulated the expression of TIF2 and promoted the interaction of EGFR with TIF2 in nasopharyngeal carcinoma. Furthermore, we demonstrated that the intact complex was linked with cyclin D1 promoter activity in an LMP1-dependent manner. The physiological functions of the intact complex were associated with cell proliferation and cell cycle progression. These findings suggest that TIF2 is a novel binding partner for nuclear EGFR and is involved in regulating its target gene expression.

Materials and methods

Cell lines and cell culture

CNE1, HNE1, and HONE1 cells are LMP1-negative nasopharyngeal squamous carcinoma cell lines. CNE1-LMP1 is a stable LMP1-integrated nasopharyngeal squamous carcinoma cell line. HNE2-pSG5 is an EBV-LMP1-negative human NPC cell line produced through transfection with the pSG5 vector into HNE2 cells. HNE2-LMP1 is a cell line with constitutive expression of LMP1 after the introduction of full-length LMP1 cDNA into HNE2 cells. Cervical cancer cells (HeLa) were purchased from the American Type Culture Collection (ATCC; Manassas, VA). MGC cell line was established from a primary poorly differentiated mucoi adenocarcinoma of human stomach. Immortalized normal nasopharyngeal epithelial cell lines (NP cells) (NP69, NP69-pLNSX and NP69-LMP1) were propagated in defined keratinocyte-SFM (KSF, Gibco, Life Technologies, Basel, Switzerland) medium supplemented with growth factors and maintained at 37°C with 5% CO2.

Plasmid constructs and site-directed mutagenesis

The pcDNA3.1-EGFR expression plasmid was constructed by cloning the entire EGFR coding fragment into the Xhol sites of the pcDNA3.1 vector. The pSG5-TIF2 vector (containing full-length hTIF2) was a generous gift from Professor Hinrich Groneneyer. The pSG5-based expression vector for wild-type LMP1, derived from the B95.8 EBV strain, was kindly provided by Dr Izumi (Brigham and Women’s Hospital). The luciferase reporters (pCD1-dLuc) used in the transfection experiments were provided by Dr Strauss and contained 3.9 kb of the human cyclin D1 promoter cloned into the multiple cloning sites of pBSK’, driving the expression of firefly luciferase (pCD1-dLuc). The EGFR motif mutants (pD1-mut-Luc) from pCD1-dLuc were generated by polymerase chain reaction (PCR) based on an overlap extension technique. The primers used for generating mutations were: 5′-GGTTCCATC-CTCTAGAGATAG-3′ and 5′-GATTTGCATTTGGATGAAAACCGGAC-3′. PCR-amplified fragments carrying the desired mutations were then cloned into Xba I sites of the pBSK’ vector. The expected mutations and the integrity of the vector were confirmed by direct sequencing (TAKARA). To monitor the transfection efficiency, pKL-SV40 (from Promega) was used as an internal control.

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Preparation of cell lysates and western blot analysis

Cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), lysed in ice lysis buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1% SDS, 5 mM dithiothreitol, 10 mM phenylmethyl sulfonylfluoride, 1 mM Na_3VO_4, 1 mM Na_3F_10 (vol/vol) glycerol, protease inhibitor cocktail tablet (Roche)) and centrifuged at 15,000 x g for 10 min after sonication. The supernatants were collected as whole cell lysates. A quantity of 50 μg of total protein was used for western blot analysis. The following antibodies were used for western blotting: mouse LMP1 monochlonal antibody (M0897, DAKO), rabbit anti-human EGFR antibody (sc-03, Santa Cruz), mouse anti-TIF2 (610984, BD Transduction Laboratories) and β-actin (A5441, Sigma).

Reverse transcription and real-time PCR

Cells were transfected with the specified siRNAs for 48 h and harvested with Trizol (Invitrogen). cDNAs were synthesized with SuperScript II (Invitrogen) according to the manufacturer’s protocol. Real-time PCR analysis was performed using the Applied Biosystems 7500 Real-Time PCR System, according to the manufacturer’s instructions. The reactions were performed in duplicate for four independent experiments; the results were normalized to actin. The primer sequences used are as follows: cyclin D1 forward 5'-TCCACCTCACCCTCTAAT-3' and reverse 5'-AGAGCCCAAAG-GCATC-3'; actin (forward) 5'-TTCCAGCCTCTCCCTG-3' and (reverse) 5'-TTCCACCTGAGGAGCAA-3'. The mean ± SEM of three independent experiments is shown.

Confocal laser analysis

Cells were cultured and fixed in 4% paraformaldehyde for 30 min. To identify the presence of EGFR and TIF2 proteins, cells were incubated with an anti-EGFR antibody (sc-03, Santa Cruz) and an anti-TIF2 antibody (BD Biosciences) and then with fluorescein isothiocyanate-conjugated anti-IgG (Santa Cruz) and Cy3-conjugated anti-IgG (Sigma). To visualize the nucleus, the cells were stained with Hoechst (1:1000). Fluorescent images were observed and analyzed with a laser scanning confocal microscope (Bio-Rad MRC-1024ES).

Co-immunoprecipitation analysis

Immunoprecipitation was performed with transfected CNE1 and CNE1-LMP1 cells. Cells were plated overnight in 100 mm² dishes (1.5 × 10⁶ cells/dish). Cells were transiently transfected with pCMV-EGFR (Santa Cruz), rabbit anti-EGFR (sc-03-G, Santa Cruz) or 2 μg of anti-GRP1 (sc-81280, Santa Cruz) and rabbit anti-GRP1 (sc-2027, Santa Cruz). Acetylated H3 (06-599, Upstate) and Acetylated H4 (06-866, Upstate) and H3 (06-755, Upstate). The chromatin reimmunoprecipitation (reChIP) assays were performed according to the manual’s protocol (Active Motif). The primers from the cyclin D1 promoter domain with the EGFR-binding region were used in the ChIP assays: 5'-TCCACCTCACCCTCTAAT-3' and 5'-AGAGCCCAAAG-GCATC-3' (181 bp).

Transient transfection and luciferase reporter assays

To study the transcriptional activity of transiently expressed pCMV-EGFR and/or pSG5-TIF2, the EGFR and/or the TIF2 expression vector (200 ng) was transfected with 0.5 μg of pCD1-Luc or 0.5 μg of pD1-mut-Luc reporter vector and the internal control plasmid pRL-SV40, as indicated. For the two-hybrid assay, Gal-TIF2, VP-EGFR and pFR-Luc (Stratagene) were transfected into cells with Lipofectamine 2000 (Invitrogen) for 48 h according to the manufacturer’s recommendations. Cells were harvested for analysis of luciferase activity using the Dual Luciferase Reporter assay (Promega) and the GloMax™ Microplate Luminometer (Promega). The luciferase reporter plasmids were co-transfected with pRL-SV40 to correct for variations in transfection efficiency. The luciferase activity was normalized to the value of pRL-SV40 activity. The results are expressed as fold induction of pCCD1-Luc activity, which was assigned a value of 1. The data represent the mean ± SD of three independent experiments performed in triplicate.

RNA interference

To reduce EGFR and TIF2 expression, we used EGFR siRNA (Sigma) and non-targeting siRNA SMARTpool (Sigma). The indicated siRNA [100 pmol EGFR siRNA and/or 25 pmol TIF2 siRNA] was transfected into cells at 30–40% confluence using Lipofectamine as recommended (Invitrogen). The cells were harvested 72 h after transfection.

To study the transcriptional activity of endogenous TIF2, cells were transiently cotransfected with pCCD1-Luc and 10nM siRNA for TIF2 or a non-coding control siRNA using Lipofectamine (Invitrogen) as described above. In some studies, cells were cotransfected with pSG5-TIF2 (500ng/24 well plate) to overcome inhibition by the siRNAs.

Cell proliferation assay

Cells were seeded at a density of 8 × 10⁴ cells/well in RPMI-1640 medium (100 μl) into 96 well plates. After a 24 h incubation, cells were transfected with plasmids or siRNA and incubated for 24–72 h. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) method, following the manual of the CellTitre 96 Aqueous One Solution Cell Proliferation assay (Promega) with a VERSAnax microplate reader (Molecular Devices). The cell survival rate was expressed as A/B × 100, where A was the absorbance value from the experimental cells and B was that from the control (untreated) cells.

Flow cytometry

Flow cytometry was used to quantify cells in each phase of the cell cycle. Cells (2 × 10⁴) were plated into 6 well plates and treated with siRNA after 24 h. Cells were harvested after an additional 72 h, washed with PBS and fixed in 70% ethanol overnight at 4°C. To detect the fluorescence intensity of certain proteins, cells were counterstained in the dark with 50 μg/ml phosphatidyl inositol and 0.1% ribonuclease A (RNase A) in 400 μl of PBS at 25°C for 30 min. Stained cells were assayed and quantified using a FACSort Flow Cytometer (Becton Dickinson).

Statistical analyses

All experiments were performed in duplicate or triplicate, and each experiment was repeated at least three times. Values were compared with basal or vehicle-treated samples. The statistical significance of differences between two groups was assessed by a two-tailed Student’s t-test. P < 0.05 was considered to represent a statistically significant difference. All the statistical analyses were performed by SPSS software (version 13.0).

Results

EBV-encoded latent membrane protein 1 upregulated TIF2 protein expression

To measure TIF2 expression in nasopharyngeal carcinoma cells, western blot analyses were performed on CNE1 and CNE1-LMP1 cells using antibodies against TIF2; the cervical cancer cell line HeLa was used as a positive control for TIF2 expression. The data presented in Figure 1A indicate that LMP1 increased the expression of TIF2. Interestingly, we did not detect differences in TIF2 mRNA levels between CNE1 and CNE1-LMP1 cells using real-time PCR analysis (Supplementary Figure S1A and S1B is available at Carcinogenesis Online). However, LMP1 increased the TIF2 mRNA levels after the treatment of MG132, a proteasome inhibitor (Supplementary Figure S1C and S1D is available at Carcinogenesis Online). To confirm TIF2 expression levels in NPC cells, we performed western blot analyses in three additional NPC cell lines, HNE2, HNE1 and HONE1, and determined that all NPC cells expressed TIF2 protein. We furthermore found that LMP1 increased TIF2 expression, as shown in Figure 1B. Moreover, we found that normal immortalized nasopharyngeal cells, NP69, also expressed TIF2 protein and that LMP1 increased the expression levels of TIF2 protein in NP69-LMP1 cells (Figure 1C). After transient transfection of LMP1 into CNE1 cells for 48 h, LMP1 induced TIF2 expression in a dose-dependent manner (Figure 1D). These data sug-
gest that EBV-LMP1 could induce the overexpression of TIF2 protein in both NP and NPC cells.

![Fig. 1. LMP1 increased TIF2 expression. Assessment of endogenous TIF2 expression and its regulation by LMP1. (A) Western blotting analysis for TIF2 in CNE1 and CNE1-LMP1 cells. HeLa cells were used as positive control for TIF2. β-Actin control was included to verify equal protein loading. (B and C) Endogenous TIF2 levels were assessed in whole cell lysates from the indicated cell lines. (D) CNE1 cells were transiently transfected with increasing amounts of LMP1-expressing plasmid for 48 h as indicated. The blot was probed with the anti-TIF2 antibody. The protein expression level was quantitated by densitometry (bottom panel).]

The oncoprotein LMP1 triggered the interaction of EGFR with TIF2

Our previous study indicated that LMP1 could trigger nuclear accumulation of the EGFR in NPC cells. To understand the interaction of nuclear EGFR with other factors, immunoprecipitation studies with anti-TIF2 antibody indicated that TIF2, a transcriptional activator, interacted with EGFR in both CNE1 and CNE1-LMP1 cells; moreover, LMP1 promoted the interaction of TIF2 with EGFR (Figure 2A). To confirm the intact complex, data in Figure 2B demonstrated that EGFR interacted with TIF2 with anti-EGFR antibody and LMP1 also promoted the interaction of EGFR with TIF2.
To examine whether TIF2 and its binding partner EGFR colocalize to the same subcellular compartments, confocal microscopic studies were performed. Both CNE1 and CNE1-LMP1 cells were dual stained with a Cy3-conjugated anti-TIF2 antibody, fluorescein isothiocyanate-conjugated anti-EGFR antibody and Hoechst 33258 and analyzed with confocal microscopy. As shown in Figure 2C, TIF2 was predominantly localized within the nucleus and was also weakly detected in the cytoplasm of CNE1 cells. In the presence of LMP1, EGFR was detected predominantly in the nucleus, which was consistent with the results of our previous study (7). The merged images indicated that TIF2 and EGFR were colocalized to the nucleus in the presence of LMP1 and EGF in HeLa cells (Figure 2C). Collectively, these data reveal that TIF2 interacts and colocalizes with EGFR.

To confirm the interaction of TIF2 with EGFR, mammalian two-hybrid assays were performed. Gal linked with TIF2 and VP16 linked with EGFR were transfected into CV-1 cells for 72 h before analysis of luciferase activity. The data indicated that the presence of LMP1 increased the interaction of EGFR with TIF2 (Figure 2D).

Direct interaction of the EGFR/TIF2 complex with the cyclin D1 promoter
We investigated whether the EGFR/TIF2 complex can bind directly to the cyclin D1 promoter region in vivo using ChIP analysis. DNA isolated from anti-EGFR or TIF2 antibody-precipitated complexes was immunoprecipitated from CNE1 and CNE1-LMP1 cells and amplified with PCR using a primer pair capable of amplifying the EGFR-binding motif sequence. As shown in Figure 3A, a band of the predicted size was detected in CNE1 cells in the EGFR-binding sites after we used EGFR and TIF2 antibodies, indicating a weak binding in the absence of LMP1. In the LMP1-positive CNE1 cells, a much stronger band was detected with both EGFR and TIF2 antibodies (Figure 3A). The results of these ChIP experiments indicate the binding role for EGFR and TIF2 in the cyclin D1 promoter.

Fig. 2. Association of the EGFR with TIF2 in the nucleus. Cells were transfected with pSG5-TIF2 for 24 h. Equal amounts of protein were immunoprecipitated (IP) with an anti-TIF2 monoclonal antibody (A) or anti-EGFR antibody (B) and were immunoblotted to detect EGFR or TIF2. (C) LMP1 induced the colocalization of EGFR and TIF2 in the nucleus. Cells were stained with the indicated antibodies. Colocalization of EGFR and TIF2 is shown as yellow in the merged image. Scale bar, 10 μm. (D) Two fusion proteins were prepared: VP-EGFR and Gal-TIF2. Luciferase activity was measured in CV-1 cells transfected with FR-luc together with plasmids expressing LMP1. Transient transfection and luciferase reporter assays were performed as described.
To confirm whether the EGFR/TIF2 complex was directly involved in binding the cyclin D1 promoter, reChIP assays were performed. We used an anti-EGFR antibody to pull down the nuclear intact complex after crosslinking; then we used anti-TIF2 or IgG to pull down the nuclear complex. In addition, we used an anti-TIF2 antibody to pull down the nuclear intact complex after crosslinking and then used anti-EGFR or IgG to pull down the nuclear intact complex. The data in Figure 3B and C illustrate that both TIF2 and EGFR simultaneously bound to the EGFR-binding site of the cyclin D1 promoter. However, LMP1 increased the binding of EGFR and TIF2 to the cyclin D1 promoter, indicating that the intact complex of TIF2 and EGFR targeted cyclin D1 directly.

To further address whether the intact complex of TIF2 and EGFR was involved with gene activation, we detected levels of histone acetylation at the EGFR-binding site near the promoter-proximal region of the cyclin D1 promoter. The data showed that both acetylation of H3 (Figure 3D) and H4 (Figure 3E) increased at least 2-fold in the presence of LMP1, indicating that histone modification by acetylation is involved in the gene activation by the intact complex of EGFR and TIF2.

Intact complex of TIF2 and EGFR transactivated cyclin D1 promoter activity

To address whether TIF2 affects gene expression in the nucleus through its association with the EGFR, we performed a gene reporter assay using the cyclin D1 promoter with wild-type luciferase (pCCD1-wt-Luc) in CNE1 cells in the presence or absence of LMP1. We transiently overexpressed TIF2 and EGFR plasmids into CNE1 cells. The wild-type cyclin D1 promoter activity was increased 8.4- and 6.4-fold after transient transfection of EGFR and TIF2, respectively, under the regulation of LMP1 (Figure 4A). Moreover, overexpression of both EGFR and TIF2 increased the cyclin D1 activity 18.3-fold, as indicated in Figure 4A. We also constructed a mutant cyclin D1 promoter with a mutated EGFR-binding site (pCCD1-mt-Luc), which abolished transcription factor binding to the EGFR-binding site. The data in Figure 4B indicate that the cyclin D1 promoter activity in the mutant construct was greatly reduced in the presence of LMP1 after transient transfection of EGFR, TIF2, or both EGFR and TIF2. Taken together, these results support the involvement of TIF2 in EGFR-induced gene expression, such as cyclin D1.

ATRS-dependent regulation of cyclin D1 promoter activity by the EGFR/TIF2 complex

The EGFR-binding sequence belongs to the AT-rich region sequence (ATRS) present in numerous genes such as the cyclin D1 promoter (9,20). To determine whether LMP1 affects cyclin D1 gene expression through the binding of the ATRS region of the promoter and, if so, whether TIF2 is the protein through which nuclear EGFR binds to the cyclin D1 gene promoter, we performed reporter assays using cyclin D1 promoter constructs with wild-type or mutated ATRS. Compared with the promoter containing wild-type ATRS, the mutated ATRS in the cyclin D1 promoter decreased the EGFR-induced luciferase activity (lane 2 of Figure 4C) and also blocked the effects of TIF2 in stimulating cyclin D1 promoter activity (lane 3 of Figure 4C). Moreover, the ATRS mutation attenuated the luciferase activity induced by coexpression of EGFR and TIF2, as shown in lane 4 of Figure 4C. In addition, we transiently transfected EGFR or TIF2 into cells together with the cyclin D1 promoter constructs; the wild-type cyclin D1 showed increased transactivation after transient transfection with EGFR and TIF2. The activity of the cyclin D1 promoter with the ATRS mutation was not stimulated by either EGFR or TIF2 (Figure 4D), suggesting that ATRS is critical for nuclear EGFR or TIF2 to turn on the cyclin D1 promoter activity. These data also indicated that the EGFR-binding site in the cyclin D1 promoter was essential for TIF2 function. Cyclin D1 with ATRS mutation was activated in the...
presence of both EGFR and TIF2 after stimulation by LMP1 (as shown in Figure 4D). However, this activation was significantly reduced compared with wild-type cyclin D1 promoter activity, indicating that the stimulatory effect of the EGFR/TIF2 complex on cyclin D1 promoter activity depends on the ATRS of the cyclin D1 promoter. In addition, EGFR and TIF2 cooperated to transactivate cyclin D1 in the presence of LMP1.

**Both TIF2 and EGFR are critical for LMP1-induced cyclin D1 gene expression**

To address whether the intact complex of EGFR and TIF2 was involved in cyclin D1 protein expression, we transiently transfected the EGFR or TIF2 construct or the combination into cells. The data shown in Supplementary Figure S2, available at Carcinogenesis Online indicate that both EGFR and TIF2 were involved in cyclin D1 expression. To further confirm that the intact complex of EGFR and TIF2 may be involved in the binding and transactivation of the cyclin D1 promoter, we knocked down EGFR or TIF2 expression with siRNA. After we depleted EGFR (Supplementary Figure S3A is available at Carcinogenesis Online) and TIF2 (Supplementary Figure S3B is available at Carcinogenesis Online), we detected cyclin D1 activity. The data shown in Figure 5A indicate that cyclin D1 promoter activity decreased by ~30% with siEGFR compared with the level obtained with siControl, whereas the activity decreased by ~50% with siTIF2 compared with the level obtained with siControl. In addition, the combination of siEGFR and siTIF2 affected cyclin D1 promoter activity. To confirm the role of TIF2 in promoting cyclin D1 promoter activity, we reintroduced TIF2 into the cells after knockdown of TIF2. The data shown in Supplementary Figure S4 is available at Carcinogenesis Online indicate that knockdown of TIF2 resulted in a decrease in
Fig. 5. Reduction of cyclin D1 expression in CNE1-LMP1 cells after treatment with EGFR and TIF2 siRNA and EGFR and TIF2 promoted cell growth. (A) Western blot analysis of cyclin D1 after depletion of EGFR, TIF2 or both. The protein expression level was quantitated by densitometry (right panel). (B) Luciferase activity in CNE1-LMP1 cells after depletion with the indicated siRNAs is indicated. The relative luciferase activity was normalized to the value of Renilla activity. The data are reported as the mean ± SEM Statistical analyses by t-test indicate *P < 0.05, **P < 0.001 versus the control siRNA. Equal numbers of vector control cells were treated with constructs of EGFR or/and TIF2, and cell numbers were determined daily with an MTT assay in CNE1 (C) and CNE1-LMP1 (D) cells. An equal number of CNE1 (E) and CNE1-LMP1 (F) cells were transfected with the indicated siRNAs, and relative cell numbers were determined daily with an MTT assay. Points indicate the mean of the absorbance readings of triplicate wells; bars indicate the SD. Similar results were obtained from three independent experiments.
Fig. 6. EGFR and TIF2 promoted cell cycle progression. Equal numbers of vector control cells in 6 well plates were treated with the indicated siRNAs, and cell cycle analysis was determined by fluorescence-activated cell sorting in CNE1 (A) and CNE1-LMP1 (B) cells. The data are presented from three independent experiments.
cyclin D1 promoter activity only in the presence of LMP1, whereas the reintroduction of TIF2 into the LMP1-positive cells restored cyclin D1 promoter activity. However, the combination of siEGFR and siTIF2 did not reduce more the mRNA levels of cyclin D1 (Supplementary Figure S3 is available at Carcinogenesis Online). Moreover, as shown in Figure 5B, we determined that the cyclin D1 expression decreased with depletion of either EGFR or TIF2, or both. Taken together, these findings demonstrate that both EGFR and TIF2 are essential for cyclin D1 expression in the presence of LMP1.

The intact complex of TIF2 and EGFR-promoted cell proliferation and cell cycle progression

To understand the physiological function of the intact complex in nasopharyngeal carcinoma cells, we performed a cell growth analysis in both CNE1 and CNE1-LMP1 cells after transient transfection with either EGFR or TIF2 or both. The data shown in Figure 5C indicated that stimulation with EGFR, TIF2 or both factors did not contribute significantly to cell growth in the absence of LMP1. However, as shown in Figure 5D, expression of either EGFR or TIF2 promoted cell growth in the presence of LMP1, and, as expected, the combination of EGFR and TIF2 expression greatly increased cell proliferation. To confirm the role of the intact complex in cell proliferation, we transiently transfected EGFR and/or TIF2 into MGC cells together with the LMP1 construct. The data shown in Supplementary Figure S6, available at Carcinogenesis Online indicate that both EGFR and TIF2 promoted cell growth, whereas the effects of the intact complexes were different, indicating that both TIF2 and EGFR were involved in promoting cell proliferation. To further confirm whether the intact complex involves in the cell viability, Figure 5E showed that only the combination of depletion of EGFR and TIF2 decreased little cell growth in the absence of LMP1, whereas Figure 5F demonstrated that all depletion of EGFR and/or TIF2 decreased dramatically in the presence of LMP1, indicating that both EGFR and TIF2 were contributed to promote cell growth. To further address how the intact complex of EGFR and TIF2 affects the cell cycle, we performed fluorescence-activated cell sorting analysis on both CNE1 and CNE1-LMP1 cells after knockdown of EGFR, TIF2 or both. The data in Figure 6A indicate that the depletion of EGFR, TIF2 or both proteins did not alter the cell cycle distribution without the stimulation of LMP1. The stimulation of LMP1 can promote cell cycle progression including S and G2/M phase, which was consistent with our previous findings (21). However, the data shown in Figure 6B demonstrated that the fraction of cells in G1 increased from 48.84% in the siControl group to 85.52%, 84.16% and 86.50% in the siEGFR, siTIF2 and the combination groups, respectively. The fraction of cells in S phase decreased from 32.13% in the siControl group to 10.39, 11.73 and 9.08% in the siEGFR, siTIF2 and combination groups, respectively. Moreover, the fraction of cells in G2/M phase was reduced from 19.03% in the siControl group to 4.09, 4.11 and 4.42% in the siEGFR, siTIF2 and the combination groups, respectively. Taken together, the intact complex of EGFR and TIF2 affects cell cycle progression through the cyclin D1 signaling pathway.

Discussion

In this study, we explored the potential function of TIF2 on gene expression in nasopharyngeal carcinoma cells, a prevalent cancer in south China. We found that LMP1 encoded by EBV upregulated the expression of TIF2 and promoted the interaction of EGFR with TIF2. We then investigated whether the intact complex was linked with the cyclin D1 promoter in an LMP1-dependent manner. Knockdown of TIF2 expression abrogated cyclin D1 promoter activity and mRNA levels; however, there was no effect on other cell cycle-associated proteins, such as CDK2 and CDK4 (data not shown). These findings suggest that TIF2 is a binding partner for nuclear EGFR and regulates target gene expression.

Increased TIF2 expression is associated with various cancers such as breast, prostate, ovarian and colorectal carcinomas (22–25) indicating that TIF2 overexpression may be linked with the growth stimulation of cancer cells. Here, we discovered that TIF2 was expressed in NPC and breast cell lines, but TIF2 did not regulate TIF2 at the mRNA level but did increase TIF2 protein levels, moreover, LMP1 increased TIF2 mRNA level in the presence of MG132, a proteosome inhibitor, indicating LMP1 may contribute to mRNA stabilities of some genes. This finding is consistent with reports from the Wilson group indicating that EGFR increases the protein levels of TIF2 but not its mRNA levels (18). However, we could not address the possibility that a coactivator like TIF2 directly affects cell cycle progression. Depletion of TIF2 in MCF-7 cells decreases cell growth, indicating that TIF2 may be linked with the cell cycle (26). Here, we found that knockdown of TIF2 expression levels decreased cell cycle progression and the activity of key cell cycle regulators such as cyclin D1. Interestingly, we did not see that depletion of the combination of EGFR and TIF2 protein levels with siRNA affected the physiological functioning, except for that seen in a reporter gene assay. The reason for this discrepancy may be due to the different techniques. The reporter gene assay detects activity in an exogenous system, whereas other assays detect the activity of endogenous proteins in the presence of the combined siRNAs. However, we did not find a significant difference after simultaneous depletion of TIF2 and EGFR in the presence of LMP1. The reason for this may be due to the assay conditions such as the cell seeding number and harvest times. In addition, our study has demonstrated that the EBV-encoded oncoprotein LMP1 not only recruited TIF2 to the target gene cyclin D1 directly but also enriched the binding of EGFR to the cyclin D1 promoter according to the ChIP and reChIP assays. These results indicate that other factors such as histone modification and chromatin remodeling may be involved in the regulation of the cyclin D1 promoter along with the regulation by LMP1.

The EGFR, a ubiquitously expressed receptor tyrosine kinase, is important in carcinogenesis (27). Nuclear translocation of the EGFR in breast cancer is usually co-expressed with Ki-67 and cyclin D1, both of which are indicators of cell proliferation (28,29). The interaction of nuclear EGFR with STAT5 is involved in the EGF-induced expression of Aurora-A, which contributes in cell cycle progression, cell survival and malignant transformation (30). Clearly, the presence of nuclear EGFR indicates an increased rate of cell proliferation and a poor survival rate for cancer patients. In addition to the nuclear EGFR, ligands of EGFR, such as EGF and transforming growth factor-β, also translocate to the nucleus (31,32). The retranslocation of the EGFR family of ligands including EGF, amphiregulin, heparin-binding EGF and β-cellulin into cetuximab-resistant cells is associated with the nuclear translocation of EGFR that is mediated by the Src family kinase (SFK) (6). Previous evidence has suggested that LMP1-positive cells establish an autocrine loop through EGF or tumor necrosis factor-α (TNFα) and their receptors (33,34), indicating that LMP1 may upregulate TIF2 protein expression (but not mRNA levels) via the autocrine loop of EGF.

Recent reports have shown that the nuclear EGFR could interact with RNA helicase A (RHA), a DNA-binding partner, after EGF stimulation in breast cancer cells. However, interaction between the EGFR and RHA is required but not sufficient to trigger cyclin D1 promoter activity (35), indicating the possibility that other factors are involved in the complex that targets genes such as the cyclin D1 promoter. Notably, the mineralocorticoid receptor has two activation functions, designated as activation function 1a (AF-1a) and AF-1b. TIF2 (a known component of the AF-2 coactivator complex) potentiates the transactivation function of AF-1b but not that of AF-1a, and RHA interacts with the AF-1a region (36). Therefore, TIF2 may directly link with RHA as well as EGFR under some conditions. Our findings also showed the interaction of TIF2 and EGFR in the presence of LMP1. Interestingly, the human DNA-protein interactor demonstrates that EGFR is a DNA-binding protein (37), which has been confirmed by gel shift assays from different groups (7,20).

The composition of regulatory complexes and the biological activities of the bound factors are dynamic and dependent on cell and response element contexts (13). TIF2 appears to have no enzymatic activities of its own, and secondary cofactors and chromatin-modi-
flying enzymes, including histone acetyltransferases and arginine and lysine methyltransferases, are recruited by TIF2 primarily to mediate transcriptional activation (38). The MOZ-TIF2 complex is associated with acute myeloid leukemia. MOZ is a MYST family histone acetyltransferase, whereas TIF2 is a nuclear receptor coactivator that associates with the CREB-binding protein. The MOZ C2HC nucleosome-binding motif is essential for transformation, whereas the acetyltransferase activity is dispensable (39,40). Because histone acetylation at both histone H3 and histone H4 is associated with gene activation, it is possible that some factors such as MOZ that are linked with histone acetylation may be involved in the process via the intact complex of TIF2 under the regulation of LMP1. It will be very interesting to address whether nuclear EGFR could directly link with the nucleosome through TIF2 or other factors, which is associated with histone acetyltransferase activity, leading to gene transcription.

In a ligand-independent pathway, numerous stimuli such as ultraviolet radiation, ionizing radiation and cisplatin treatment also result in the accumulation of nuclear EGFR, contributing to acquired treatment resistance (8,41,42). Nuclear EGFR may be an important molecular determinant of resistance to cetuximab therapy (6). Nuclear EGFR is required for the modulation of cisplatin and ionizing radiation-induced repair of DNA damage through the interaction of nuclear EGFR with DNA-damage repair complexes (43,44). EGFR–DNA-PKcs binding is induced by cisplatin and ionizing radiation but not by EGFR nuclear translocation, indicating that the EGFR subcellular distribution can modulate DNA repair kinetics with implications for the design of EGFR-targeted combinational therapies (43). Radiation therapy is a key strategy for NPC therapy; it would be interesting to determine the value of treating radiation-resistant tumors with a knockdown of LMP1 in combination with inhibition of nuclear EGFR.

This study has demonstrated that the nuclear EGFR could cooperate with TIF2 to target the promoter region of cyclin D1 and increase its expression in cancer cells. The intact complex plays a functional role in cell proliferation and cell cycle progression. These results demonstrate a novel link between the activated EGFR in the nucleus and coactivators in cell cycle progression.

Supplementary material

Supplementary Figures 1–6 can be found at http://carcin.oxfordjournals.org/

Funding

The Fundamental Research Funds for the Central Universities (2011JQ019), the National Basic Research Program of China (2011CB504300), the Hunan Natural Science Foundation of China [12JJ1013], the Program for New Century Excellent Talents in University (NCET-07-0863); the Fok Ying Tung Education Foundation (111037), and the National Natural Science Foundation of China (81171881, 30772482).

Acknowledgements

We would like to thank the lab for critical discussions of this article. Y.T. and Y.C. designed the project; S.Y., Y.J., Y.X., Y.B., X.C. and L.X. performed experiments; S.Y., Y.T. and Y.C. analyzed data; S.Y. and Y.T. wrote the article.

Conflict of Interest Statement: None declared.

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Received January 2, 2012; revised March 19, 2012; accepted April 6, 2012