ORIGINAL ARTICLE

UPREGULATION OF DISCOIDIN DOMAIN RECEPTOR 2 IN NASOPHARYNGEAL CARCINOMA

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Abstract: *Background.* Nasopharyngeal carcinoma (NPC) is associated with Epstein-Barr virus (EBV) and has high meta-static potential. Discoidin domain receptors (DDR1, DDR2) are receptor-type tyrosine kinases activated by collagen. Their ability to induce expression of matrix metalloproteinase is related with tumor invasion. Therefore, we aim to investigate DDRs gene expression and its regulation in NPC.

Methods and Results. By use of real-time quantitative poly-merase chain reaction (Q-PCR), DDR2 gene expression but not DDR1 was significantly higher in primary and metastatic NPC. DDR2 was predominantly distributed in NPC tumor cells rather than in infiltrating lymphocytes. EBV Z-transactivator (Zta) trans-fection may distinctly elevate DDR2 level. Furthermore, data from reporter assay indicate that Zta could transactivate DDR2 pro-moter activity, suggesting the possible upregulation mechanism.

Conclusion. DDR2 was differentially upregulated in NPC and modulated by EBV Zta protein. DDR2 may play a role in NPC invasion and serve as a diagnostic and therapeutic target. © 2007 Wiley Periodicals, Inc. *Head Neck* **30:** 427–436, 2008

Keywords: discoidin domain receptor gene family; discoidin domain receptor 2; nasopharyngeal carcinoma; Epstein-Barr virus; Z-transactivator

Nasopharyngeal carcinoma (NPC) is a prevalent malignancy in Southeast Asia, southern China, and Taiwan.[1] Characteristically, NPC is different from other head and neck carcinomas not only in terms of their epidemiology, clinical presentation, biological markers, carcinogenic risk factors, prognostic factors, treatment and outcome, but also in its strong association with the presence of the Epstein-Barr virus (EBV) and a high inci-dence of metastasis.[2,3] To date, the underlying mo-lecular mechanism of NPC tumor behavior remains unclear. Nevertheless, unlike in most carcinomas, mutations in well-defined tumor sup-pressor genes, eg, retinoblastoma susceptibility genes (Rb) and p53, and other oncogenes are less

frequent in NPC.[4–6] One possibility is that EBV-encoded proteins may directly influence the normal cellular biology in causing tumor formation.[7]

Among all signaling cascades in the study of cell physiology or carcinogenesis, the role of tyrosine kinase family becomes more and more important. The protein tyrosine kinases are a large multigene family involved especially in the regulation of cell to cell signals concerning growth, differentiation, adhesion, motility, and apoptosis.[8] Tyrosine kinases have been demonstrated to play crucial roles in the development of many disease states.[9] In the pathophysiology of gastrointestinal stromal tumors, genetic mutations in Kit genome lead to a gain-of-function of this tyrosine kinase receptor and result in oncogenesis.[10] Deregulated protein tyrosine kinase such as Abl is also considered as a key pathogenic event in human hematological malignancies.[11]

Notably, discoidin domain receptors (DDRs) belong to 1 subgroup of receptor tyrosine kinases (RTKs) and distinguish from other members by a discoidin homology repeat at their extracellular domains that mediate intercellular adhesion.[12] Considering most RTKs are activated by soluble ligands present in the blood or other body fluids, DDR1 and DDR2 (also called TKT, TRK-related tyrosine kinase) of DDR subfamily are unusually activated by an extracellular matrix protein, triple-helical collagen.[13] Differences between DDR1 and DDR2 are their relative affinities for collagens and tissue specificity. DDR1 is activated by type I, II, III, V, XI collagens, DDR2 is activated mainly by type I and III collagens.[14] Both DDRs are widely expressed in human and mouse tissues, but their distribution is mutually exclusive.[15] DDR1 is expressed primarily in epithelial tissues and DDR2 is found in mesenchymal cells.[15] Many recent studies have suggested that DDRs can regulate cell proliferation and extracellular matrix remodeling mediated by matrix metalloproteinase (MMP) activities during normal development or pathological conditions.[16–21] DDR1 and DDR2 may regulate collagen turnover in blood vessels, and DDR2 expression is increased by cyclic mechanical stretch in cultured rat vascular smooth muscle cells.[22,23] DDR1 has been implicated in the growth of some epithelial neoplasms, including breast cancer.[24] In 1 recent study, DDR2 was found to be differentially expressed in Hodgkin's lymphoma and anaplastic large cell lymphoma confirmed in primary tumor tissue by immunohistochemistry and/or reverse transcriptase polymerase chain reaction (RT-PCR).[25]

In our previous study utilizing a newly developed kinase display assay, DDR2 was found to be upregulated by EBV-encoded Z-transactivator (Zta) at both RNA and protein levels in a cell culture system.[26] Concurrently, we also have found that MMP-1, a DDR2 downstream effector which could promote cell migration, was significantly upregulated in NPC biopsies and in Zta-expressing cells.[27] Therefore, the main purpose of this study was to extensively investigate DDRs gene expression in NPC biopsies and other control tissues and to elucidate the relationship between DDRs family and Zta of EBV in NPC tumor. Furthermore, we attempted to explore the regulatory mechanism of Zta-upregulated DDR2 by promoter activity assay.

MATERIALS AND METHODS

Tissue Sources. NPC biopsy specimens included 35 primary tumors (NPC) and 22 metastases (NPC meta), all of which were pathologically diagnosed as either differentiated nonkeratinizing or undifferentiated types of NPC. Twenty cases of other head and neck squamous cell carcinoma biopsies (2 oral cancers, 5 buccal cancers, 1 oropharyngeal cancer, 6 tongue cancers, 4 hypopharyngeal cancers, 2 laryngeal cancers) were served as tumor control tissues. Nontumor tissues included 26 samples of nasopharyngeal lymphoid hyperplasia and 17 samples of nasal epithelial cells, all of which were pathologically shown to have an absence of tumor cells. Primary cultures of normal epithelial cells were achieved through mildly digesting nasal polyps or turbinates with 0.1% pronase (Roche, Indianapolis, IN) at 4°C overnight and then seeding detached epithelial cells onto a culture dish coated with collagens (Roche) as in our previous study.[28] The cells were grown in keratinocyte medium supplemented with 5 ng/mL human recombinant epithelial growth factor, 50 μg/mL bovine pituitary gland extract, and 5% fetal bovine serum (Invitrogen, Carlsbad, CA). Epithelial characteristics of the cultured primary cells were confirmed, in that more than 99% of the cells expressed cytokeratins. All biopsy samples were obtained from the Department of Otolaryngology, National Taiwan University Hospital. The sections of paraffin-embedded NPC and other control tissues were provided by the Department of Pathology, National Taiwan University Hospital. All procedures were in accordance with the ethical standards of the

Table 1. Nucleotide sequences of primers and probes used in Q-PCR and PCR.

Target genes	5′ to 3′ nucleotide sequences
Q-PCR	
DDR1	
Forward primer	ACTCAACACGGTGTGAATCACAC
Reverse primer	TCACTGGCTTCCCCTGGA
Probe	CAGCTGCCCCTCCCTCAGGGA
DDR2	
Forward primer	AGTCAGTGGTCAGAGTCCACAGC
Reverse primer	CAGGGCACCAGGCTCCATC
Probe	CCAAATATGGAAGGCTGGACTCAGAAG
Zta	
Forward primer	AGCAGCCACCTCACGGTAGTG
Reverse primer	AATCGGGTGGCTTCCAGAA
Probe	CAGTTGCTTAAACTTGGCCCGGCA
PCR	
DDR2	
Forward primer	GCGCCATGCAGGAGGTCATG
Reverse primer	CCACTCTCATACACACATTCA
Zta	
Forward primer	TTCCACACAGCCTGCACCAGTG
Reverse primer	GGCAGCAGCCACCTCACGGT
DAD-1	
Forward primer	GCAGTTATGTCGGCGTCGGTAG
Reverse primer	GTTCTGTGGGTTGATCTGTATTC

Abbreviations: Q-PCR, quantitative polymerase chain reaction; DDR1, discoidin domain receptor 1; DDR2, discoidin domain receptor 2; Zta, Z-transactivator; PCR, polymerase chain reaction; DAD-1, defender against death 1 protein.

committee on human experimentation of College of Medicine, National Taiwan University.

Isolation of Cell Subpopulations from Nasopharyngeal Carcinoma. Epithelial tumor cells, CD4$^+$ T cells, and CD8$^+$ T cells were isolated from 7 fresh NPC biopsies using specific monoclonal antibody-coated paramagnetic beads.[29] The purity of each bead-selected subpopulation was confirmed by immunoflow cytometry and proved to be >95% as previously reported.[29]

RNA Extraction. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The quality and quantity of total RNA were determined by gel analysis with Sybre green staining and spectrophotometry.

Real-Time Quantitative Polymerase Chain Reaction. Complementary DNA (cDNA) was synthesized from 1 μg of DNase I-treated (Gibco BRL, Gaithersburg, MD) total RNA, mixed with random hexamers (Gibco BLR), and heated to 70°C for 10 minutes. Thereafter, the reaction mixture was incubated with deoxyribonucleoside triphosphates (dNTPs), dithiothreitol, and RT reaction buffer at room temperature for another 10 minutes. Finally,

SuperScript II RNase H$^-$ reverse transcriptase (Gibco BLR) was added to generate 20 μL of cDNA at 42°C for 50 minutes.

The quantitative (Q)-PCR was performed as described in our previous publication.[28] Each sample was tested in duplicate. A positive control of Zta stable clone was performed in every set of reaction to confirm the efficacy and efficiency of each round of Q-PCR amplification. The primers and probes of each target gene utilized in Q-PCR were designed using Primer Express software (Applied Biosystems, Foster City, CA), and the sequences are listed in Table 1. The detection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts in each sample served as an internal control in Q-PCR to standardize the amount of input cDNA. The probe and primers of GAPDH used in Q-PCR were purchased from a commercial source (TaqMan GAPDH Control Reagent Kit; Applied Biosystems). The relative amounts of tested transcripts (40-Ct) were calculated as described previously.[28]

Polymerase Chain Reaction. A 25 μL reaction mixture contained 2 μL of cDNA, 1× PCR reaction buffer, 0.2 mM dNTPs, 0.4 μM of primers, and 1 U of Dynazyme^{TM} II DNA polymerase (Finnzymes, Oy, Finland). DDR1 transcripts were amplified at 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute for 30 cycles. For DDR2, the amplification was optimized at 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute, which was carried out for 30 cycles. Also, the Zta transcripts were determined by PCR accomplished at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, total to 25 cycles. Besides, defender against death 1 (DAD-1) protein, an internal control, was included in each assay and performed 25-cycle amplification of 94°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds. The primer sequences for PCR are listed in Table 1, and the DDR1 PCR primers are the same as that of Q-PCR.

Immunohistochemical Staining. After 3 rounds of deparaffinization in xylene for 10 minutes each, the sections were rehydrated in graded alcohol. To retrieve antigen, these sections were subsequently brought to microwave boiling in 10 mM sodium citrate buffer (pH 6.0) for 10 minutes and then allowed to cool at room temperature for another 30 minutes. The sections were then treated with 0.3% hydrogen peroxide for as long as 30 minutes, followed by blocking of endogenous

avidin/biotin (Zymed Laboratories, San Francisco, CA). After blocking in normal goat serum (from Histostain-Plus Kit; Zymed Laboratories) for 1 hour, the sections were incubated with DDR2 polyclonal antibodies (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight.[29] The following staining procedures were performed according to the manufacturer's instructions (Zymed Laboratories). Finally, the sections were counterstained with hematoxylin. The staining of placental tissue was served as a positive control, which has been reported as a typical DDR2 expressive source,[26] and NPC biopsy sample staining with same procedure but omitting primary antibody was served as a negative control.

Western Blotting. Cell extracts were collected in lysis buffer (3% SDS, 2M urea, and 2% 2-mercaptoethanol). Twenty microgram of protein from each extract was separated in a 10% SDS-polyacrylamide gel and electroblotted onto Hybond™-C membrane (Amersham Biosciences, Uppsala, Sweden). The membrane was then blocked in blocking buffer [4% milk in 0.01M Tris-HCl (pH 7.4), 0.15M NaCl, and 0.2% Tween-20] at room temperature for 2 hours. Immunoblotting was performed with antibodies against DDR2 (1:100 dilution; Santa Cruz Biotechnology), Zta (1:20 dilution),[26] or anti-β-actin (1:10,000 dilution; Sigma, St. Louis, MO) at 4°C overnight. After washing with Tris-buffered saline containing 0.2% Tween-20, the blots were probed with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies. Finally, bands were revealed by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

Reporter Gene Assay. The luciferase reporter plasmid driven by DDR2 promoter (pDDR2-Luc) was constructed by inserting nucleotides −1423 to +10 of the DDR2 promoter into pGL2-Basic vector (Promega, Madison, WI). Nucleotides −221 to +12 of Zta promoter was also cloned into pGL2-Basic vector to generate luciferase reporter plasmid designated Zp-Luc and served as control plasmid.[30] Both reporter plasmids were transfected transiently with Zta- and GFP- (pEGFP-C1; Clontech, Mountain View, CA) expression plasmids into NPC-TW01 cells. Transfections were performed following the manufacturer's instructions (TransFast Transfection reagent; Promega). The transfected cells were trypsinized and subjected to determine serially for GFP (internal control)

fluorescent intensity and Firefly luciferase activity according to manufacturer's protocols (Bright-Glo Luciferase Assay Kit; Promega). The activities of Firefly luciferase relative to GFP intensities were calculated and were indicated after being normalized to those of control vectors. The relative transcriptional activity was shown as folds of induction.

Statistical Analysis. The means and standard errors of Q-PCR data among all categories of tissues were calculated, and the differences between them were determined by t test, where $p < .05$ is regarded as significant. All statistical analytic works were accomplished with Statistical Analysis Software.

RESULTS

Expression of DDRs in Nasopharyngeal Carcinoma, Nasopharyngeal Carcinoma Meta, and Other Control Tissues. By using real-time Q-PCR, DDR1 transcripts were found to be expressed at similar levels in all samples tested, ie, nasal epithelial cells, nasopharyngeal lymphoid hyperplasia specimen, NPC, NPC meta, and head and neck tumor tissues ($p > .1$) (Figure 1A). However, DDR2 expression in these tissues varied considerably. Homogenous nasal epithelial cells isolated from nasal turbinates or polyps were served as nontumor control. They expressed almost no DDR2 transcripts (Figure 1B). Nasopharyngeal lymphoid hyperplasia specimen contained mixed cellular population of lymphocytes and epithelial cells as in NPC biopsy was served as another nonmalignant control. Of note, significant expression of DDR2 was shown in 3 tumor groups (NPC, NPC meta, and head and neck tumors). Among them, DDR2 expression was significantly higher in NPC and NPC meta compared with nasopharyngeal lymphoid hyperplasia specimen ($p < .0001$) (Figure 1B). Furthermore, the relative levels of DDR2 transcripts in NPC were also higher than those of head and neck tumors ($p < .0005$) (Figure 1B). NPC tissues characteristically expressed high level of DDR2 transcripts.

Distribution of DDR2 Transcripts in Nasopharyngeal Carcinoma Tissues. Pathologically, NPC histology shows epithelial tumor nests scattered around heavily infiltrating lymphocytes. These lymphocytes comprised mainly CD4$^+$ T cells and CD8$^+$ T cells.[29] To determine which cell type(s) may express DDR2 in NPC specimens, the epithelial
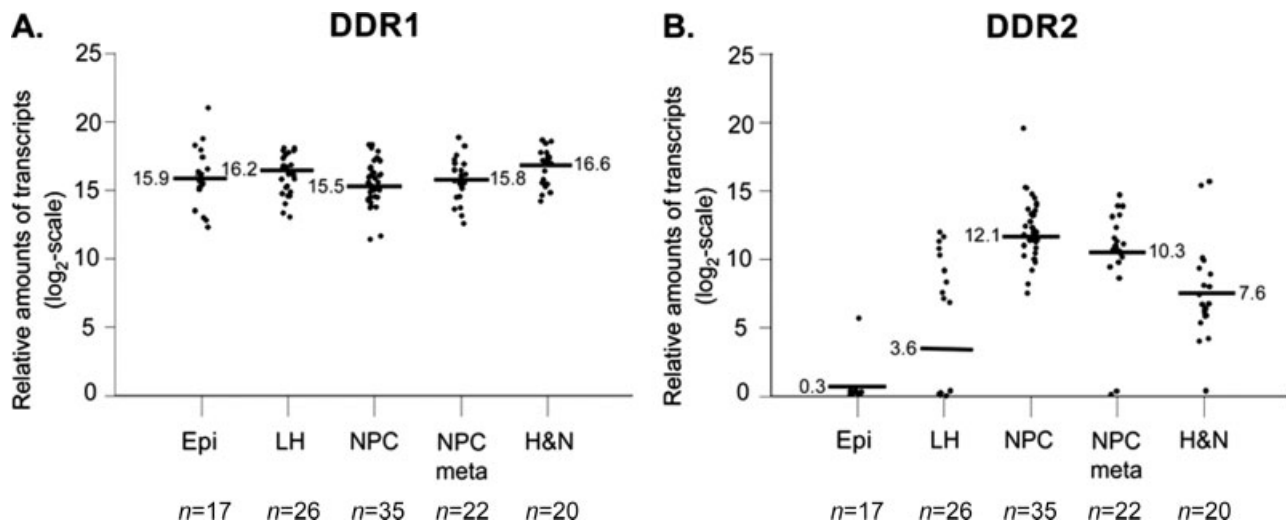
FIGURE 1. Examination of the amounts of discoidin domain receptor (DDR)-1 and DDR-2 transcripts in nasal epithelial cells (Epi), nasopharyngeal lymphoid hyperplasia control tissues (LH), primary NPC, NPC metastatic tissue (NPC meta), and head and neck squamous cell carcinoma (H&N) with real-time quantitative polymerase chain reaction (Q-PCR). The *y*-axis plotted in log$_2$-scale depicts the relative amounts of transcripts of DDR1 (**A**) and DDR2 (**B**), which had been standardized against the GAPDH internal control as described in Materials and Methods section. The horizontal bars indicate the mean values.

tumor cells, CD4$^+$ T cells, and CD8$^+$ T cells were separated from fresh NPC specimens using antibody-coated paramagnetic beads.[29] Then, real-time Q-PCR was applied to determine the relative levels of DDR2 transcript in each cell population. The result showed that DDR2 was constantly expressed in epithelial cells in all 7 biopsies tested. Less frequently, DDR2 was expressed in 4 and 2 cases of CD4$^+$ and CD8$^+$ cells, respectively. Considering normal nasal epithelial cells contained almost no DDR2 transcripts (Figure 1B), this evidence indicated that DDR2 transcripts appeared predominantly in epithelial tumor cells rather than in CD4$^+$ T cells or CD8$^+$ T cells (Figure 2).

Immunohistochemical Staining of DDR2 Proteins in Nasopharyngeal Carcinoma Tissues. Besides the evidence demonstrating DDR2 gene expression upregulated in NPC (Figure 1B) and distributed mainly in epithelial tumor cell (Figure 2) at the transcriptional level, immunohistochemical staining was also performed to determine the distribution of DDR2 protein in NPC biopsies. The result showed that positive signals of DDR2 were exhibited both in the cytoplasm and plasma membrane of tumor cells in 9/15 of NPC biopsy sections. A typical staining section was shown in Figures 3A and 3B. A lesser extent of staining was shown in the infiltrating lymphocytes that were also demonstrated in Figure 2 showing some infiltrating T-cells may still expressed DDR2 molecules. These

results were well in accordance with the finding that DDR2 was expressed mainly in NPC epithelial tumor cells.
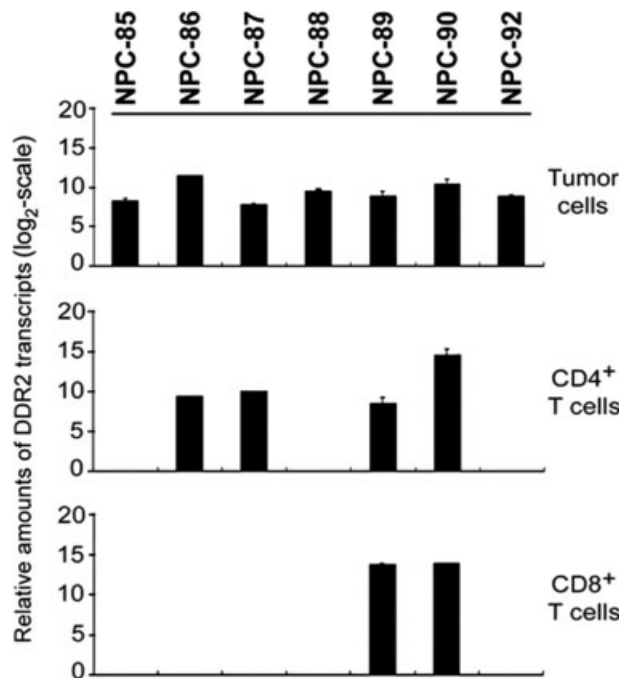
FIGURE 2. Distribution of DDR2 transcripts in NPC tissues. Cellular subpopulations in NPC tissues, including epithelial tumor cells, CD4$^+$ T cells, and CD8$^+$ T cells, were isolated from fresh NPC specimens using specific antibody-coated paramagnetic beads. Total RNA was purified from each subpopulation and the amounts of DDR2 transcripts were evaluated by real-time quantitative polymerase chain reaction.
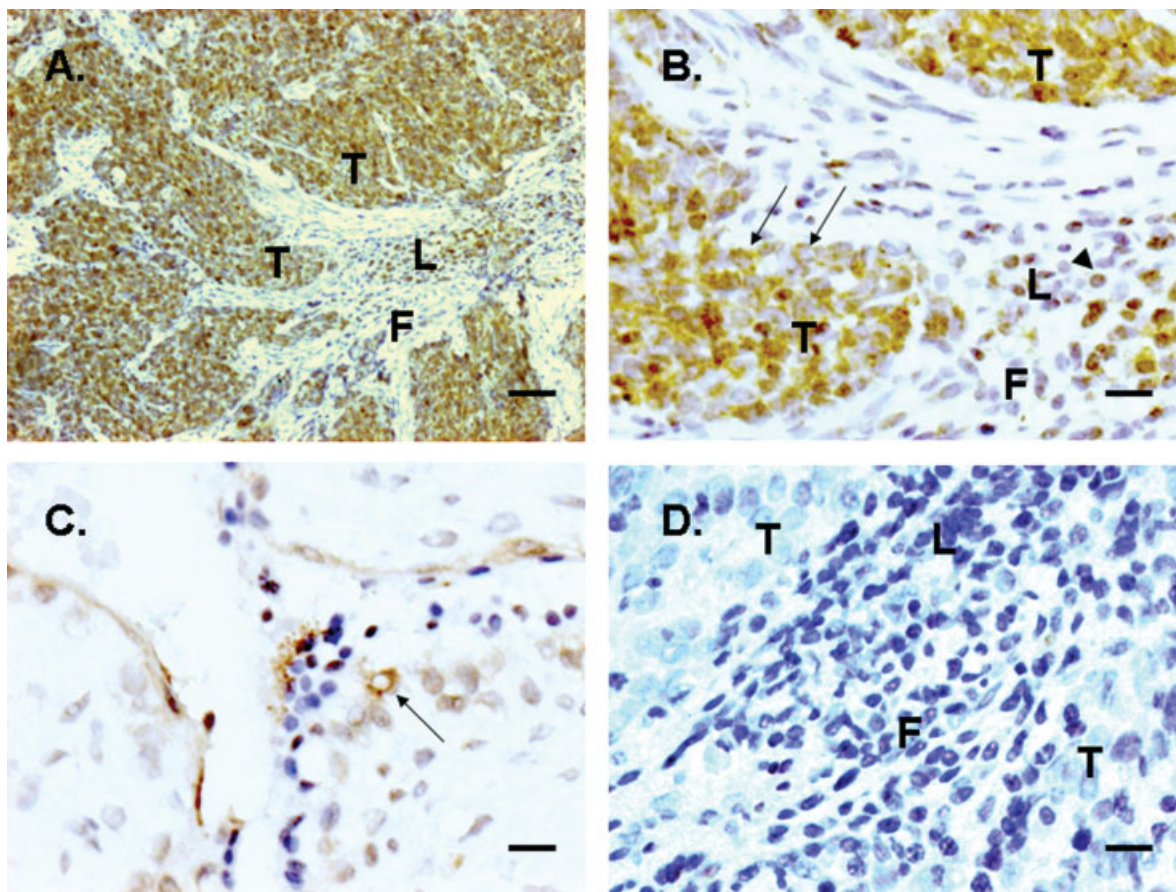
FIGURE 3. Demonstration of DDR2 proteins in NPC biopsies by immunohistochemical staining. Deparaffinized sections of NPC biopsy and placental tissue were stained with polyclonal antibody against DDR2 protein. (**A**) Positive, dark brown signals were clearly seen in tumor area (T) but not in fibrous stroma (F). (**B**) Under higher magnification, the dark brown signals were present mostly in cytoplasm and plasma membrane of tumor cells (long arrows) and, to a lesser extent, in lymphoid cells (L) (arrowhead). (**C**) DDR2 staining was performed in placental tissue. Positive staining was indicated by long arrow. (**D**) Negative control was performed by omitting primary antibody during staining procedure in NPC tissue. Bars indicate 100 μm in A, 25 μm in B, C, D. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

EBV Zta Gene Correlated with DDR2 Gene Expression. NPC is a well-known EBV-associated malignancy. Several EBV gene products, including EBV nuclear antigen (EBNA)-1, latent membrane protein (LMP)-1, LMP-2A, and Zta have been demonstrated to be present in NPC biopsies.[2] By use of real-time Q-PCR, we investigated the correlation of transcripts quantities between these viral gene and DDR2 gene expression in 35 NPC biopsies. The results showed that only the expression of Zta transcripts was significantly correlated with DDR2 transcripts ($p = .0027$, Figure 4A). Other EBV-related genes, ie, EBNA-1, LMP-1, and LMP-2A, did not show any correlation with DDR2 expression in NPC (data not shown). By use of PCR and western blotting, we also demonstrated that both DDR2 transcripts and proteins

were correspondingly increased in a Zta stable clone (Zta-expressing RHEK cells). Specifically, stable expression of Zta was noted to be related with DDR2 transcript (Figure 4B) and protein (Figure 4C) expression, but not with its family member DDR1 gene products. These results provided evidence that EBV Zta gene have effects on upregulating DDR2 gene expression in both in vivo and in vitro conditions.

EBV Zta Gene Induces DDR2 Promoter Activity. To further reveal the regulatory mechanism of EBV Zta on DDR2 gene transcription, a DDR2 promoter-derived luciferase reporter plasmid (pDDR2-Luc) was constructed. Upon Zta cotransfection, the luciferase activity driven from Zta pro-
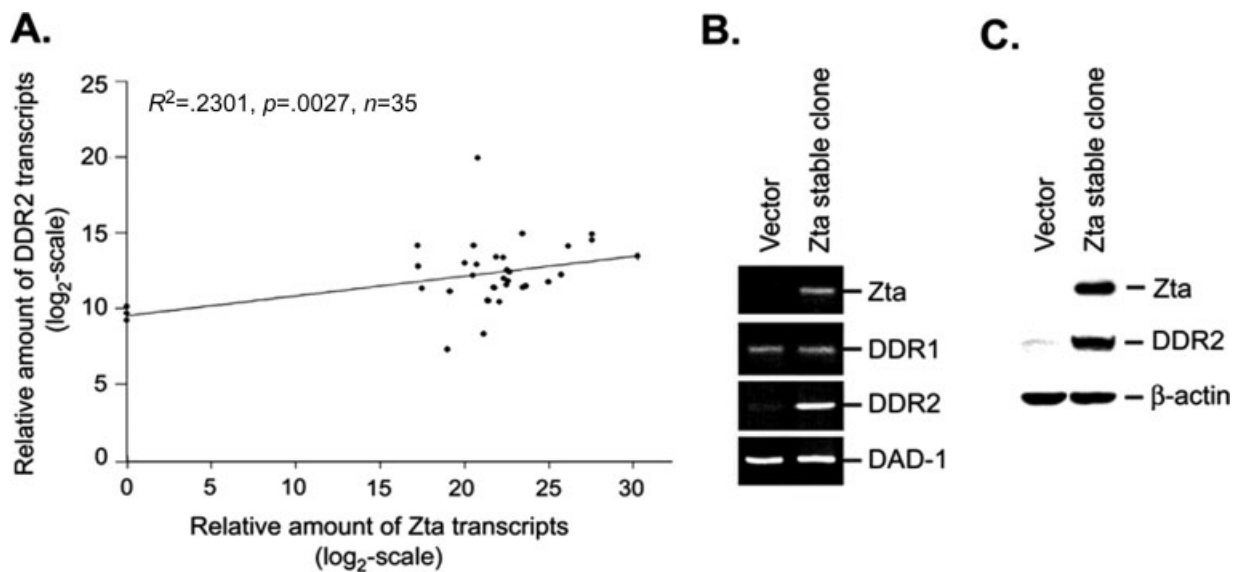
FIGURE 4. Relationship of Zta with DDR2 expression. (**A**) Real-time quantitative polymerase chain reaction (Q-PCR) was performed to detect the expression of DDR2 and Zta transcripts in 35 cases of NPC biopsies. Correlation of Zta and DDR2 transcripts amount was statistically significant (R^2 = .2301, p < .005). (**B**) Expression of DDRs in Zta transfection stable clone. RNA extracted from Zta-expressing RHEK cells (Zta stable clone) and control cells (vector) were subjected to RT-PCR analysis using Zta, DDR1, DDR2, and DAD1-specific primers. The detection of DAD-1 transcript was provided as an internal control. (**C**) Determination of DDR2 protein expression by western blotting. Twenty microgram cell extracts of Zta-expressing RHEK and control vector cell were immunoblotted with Zta and DDR2-specific antibodies. Following the horseradish peroxidase-labeled goat anti-mouse or goat anti-rabbit antibodies incubation, the blots were finally visualized by enhanced chemiluminescence. The measurement of β-actin protein expression was included as an internal control. DDR2 was obviously upregulated in Zta stable clone cells in both transcriptional and translational levels.

moter (Zp-Luc) was elevated approximately 4-fold compared with vector control (Figure 5) as predicted.[31] Impressively, pDDR2-Luc had an 8-fold increase in luciferase activity when Zta was coexpressed (Figure 5), suggesting a mechanism that
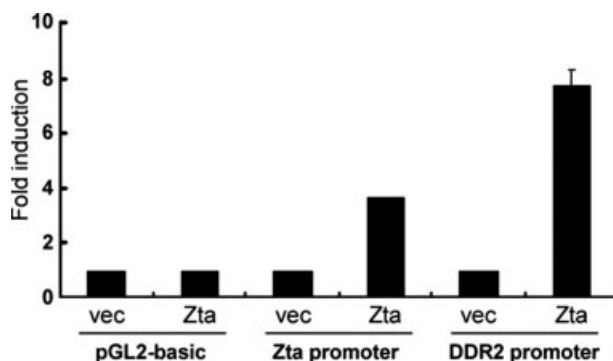
FIGURE 5. Transactivation of DDR2 promoter activity by Zta. NPC-TW01 cells were transfected with 0.4 μg GFP, 1.6 μg Zta, and 0.4 μg of pDDR2-Luc or Zp-Luc plasmids as described in Materials and Methods section. Rc/CMV and pGL2-basic were the control vectors of Zta and pDDR2-Luc, respectively. Twenty-four hours following transfection, the cells were harvested for assessing luciferase activities. The results are expressed as folds of induction of luciferase activity from duplicate experiments. The representative data from 3 independent experiments are shown.

Zta may upregulate DDR2 expression through induction of DDR2 promoter activity.

DISCUSSION

Considering 1 of the crucial biological functions of RTKs is to modulate the expression of E-cadherin, β-catenin, as well as of MMPs, RTKs are often suggested to be involved in cell adhesion, extracellular matrix modification in physiological condition and related with tumor invasiveness, and metastasis in cancer biology.[9,19,20] In addition to DDR2, only 3 types of RTK have been investigated in NPC until now, ie, epidermal growth factor receptor (EGFR), c-erbB-2, and Met. These RTKs are all overexpressed in NPC tumors, and their expression is correlated with severity of diseases.[32–34] Among them, EGFR and Met were reported to be regulated by EBV latent products LMP1, and C-erbB-2 by EBNA1.[32,35,36] Statistically, EGFR was strongly expressed in NPC tissues of patients with stages III and IV tumor.[32] The c-erbB-2 gene amplification was found to be correlated with clinical tumor stage in NPC.[33] Additionally, Met protein expression is higher in NPC biopsies and correlated with patients' survival duration.[34] All these data implied the impor-

tance of these RTK in the pathogenesis of NPC, and led us to explore the involvement of other RTK in NPC.

The DDR subfamily of the RTK gene family is the main target of this study and has only 2 members, ie, DDR1 and DDR2. DDR1 has an essential role in mammary gland development and has been shown to be overexpressed in breast carcinoma and other tumors such as ovary carcinoma, and esophageal carcinoma.[37] In breast cancer, DDR2 is present in stromal cells surrounding highly invasive DDR1-positive tumor cells.[38] This is in consistent with normal tissue distribution of DDRs that DDR1 expressed in epithelial cells and DDR2 in mesenchymal cells.[15] Nevertheless, our previous study demonstrated that the DDR2 was found to be upregulated in epithelial cell line by EBV immediate early gene Zta in in vitro condition.[26] Reasonably, the first question we addressed was whether both DDR gene transcripts were present in NPC tissues in vivo. Results showed that DDR1 was equally expressed in all tissue samples tested by real-time Q-PCR, including nontumor control tissues, nasopharyngeal lymphoid hyperplasia specimen, nasal epithelial cells, and 3 kinds of tumor biopsies, NPC, NPC meta, and head and neck tumors (Figure 1A). On the contrary, we demonstrated unique overexpression of DDR2 in NPC biopsies than in other control tissues (Figure 1B). Therefore, other than previous study showing that DDR2 was upregulated by EBV Zta in epithelial cell lines in vitro, we further provided evidence that DDR2 but not DDR1 was also upregulated in an EBV associated disease-NPC tissue in vivo. Although the common argument is that NPC biopsy specimen is a mixed cell population of epithelial tumor cells and infiltrated nonmalignant lymphoid cells, we separated apart these cell types and demonstrated that the expression of DDR2 was predominantly distributed in epithelial tumor cells rather than in $CD4^+$ and $CD8^+$ T lymphocytes (Figures 2, 3A, and 3B). Characteristically, the expression level of DDR2 transcripts was higher in NPC and NPC meta tissues than in various head and neck squamous cell carcinomas. Besides, DDR2 is almost absent in normal nasal epithelial cells. These results emphasized that the overexpression of DDR2 was found in head and neck epithelial cancers, especially in NPC tumor cells (Figure 1B).

In NPC tissues, the expression of Zta was positively correlated to DDR2 transcripts by use of real-time Q-PCR (Figure 4A). In addition, Zta was also shown to upregulate DDR2 at both transcriptional and translational levels in RHEK cells (Figures 4B and 4C). This evidence indicates that Zta may contribute to the upregulation of DDR2 in both in vivo and in vitro conditions. Moreover, by using reporter gene assay, we also demonstrated that transfection of Zta may upregulate DDR2 expression through induction of DDR2 promoter activity (Figure 5). Searching from the sequence of DDR2 promoter region, we have identified a Zta responsive element that may be responsible for the Zta effect on the DDR2 expression.[39,40] Although the detailed mechanism is still not clear, Zta evidently increased the transcriptional activity of DDR2 gene and resulted in the upregulation of DDR2 in NPC.

The consequences of upregulation of DDR2 gene products in NPC may be predicted by its well-known biological activities. DDR2 is reported to interact with collagen. Prolonged stimulation of DDR2 is associated with the upregulation of MMP-1 expression.[13] DDR2 also plays an important role in mediating hepatic stellate cells and fibroblast migration, proliferation by MMP-2 dependent mechanisms.[19,20] Activated DDR2 has been noted to induce the expression of MMP-1, MMP-2, and MMP-13.[41,42] Subsequently, it may have potential to degrade the major interstitial connective tissue (collagens I–III) and promote tumor migration and metastasis.[15] Besides, we have observed that MMP-1 is significantly upregulated in NPC biopsies and Zta-expressing cells and have demonstrated the involvement of MMP-1 in cell mobility and tumor metastasis by scrape-wound migration assay and 3-dimensional collagen assay.[27] In parallel, 1 of the characteristics of NPC is its higher regional metastatic potential to cervical lymph nodes and distant metastasis than other head and neck cancers.[1] Collectively, abundant DDR2 expression of both NPC and NPC meta tissues in comparison with other control tissues as shown in this study may indicate that DDR2 plays some role in NPC tumorigenesis and especially in tumor cells metastasis.

In conclusion, our studies support that EBV gene product Zta is able to activate DDR2 expression and thus facilitates NPC proliferation, migration, and metastasis. These findings might elucidate the biological role of DDR2 in NPC and contribute in the early diagnosis and prediction of metastasis. Furthermore, the development of selective inhibitors that can block or modulate kinase pathways is now widely considered a promising approach for drug development such as

Bcr-Abl in chronic myelogenous leukemia and c-Kit in gastrointestinal tumors.[43] It may even be applied in the therapeutic application in the future.

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