NF-κB induces abnormal centrosome amplification by upregulation of *CDK2* in laryngeal squamous cell cancer

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Abstract. Centrosome amplification can drive chromosomal instability (CIN) which is a major source of tumor initiation. The present study aimed to investigate the impact of nuclear factor kappa B (NF-κB) on centrosome amplification of Hep-2 cells. Immunofluorescence was performed to display centrosomes. BAY11-7082 was used as an inhibitor of NF-κB to assess the inhibition of centrosome amplification, and cyclin-dependent kinase 2 (*CDK2*), ensuring cell cycle cycle coordination with centrosome cycle was detected by Western blotting. Furthermore, a 1556-bp fragment of the *CDK2* promoter was analyzed using the TRANSFAC-TESS software. Luciferase assay, including a series of truncated *CDK2* promoters and site mutations, was carried out to determine NF-κB binding sites in the *CDK2* promoter. Electrophoresis mobility shift and chromatin immunoprecipitation assays were applied to confirm whether NF-κB indeed binds to the 5'-promoter region of the *CDK2* gene. To reveal the clinical significance of *CDK2* expression in laryngeal squamous cell cancer, mRNA and protein levels were assessed by RT-PCR and Western blotting, respectively. We found that the transcription factor NF-κB plays a role in centrosome amplification in Hep-2 cells. Centrosome amplification is reduced by inhibition of the NF-κB pathway. Moreover, expression of the p65 subunit of NF-κB is sufficient to promote centrosome amplification and increase in *CDK2* protein levels. We further identified a functional NF-κB binding site located in the *CDK2* promoter. Single mutation of the NF-κB site III (construct mutIII) however resulted in 76±5% ($p<0.01$) luciferase activity reduction. Electromobility shift assays and chromatin immunoprecipitaton results suggest that NF-κB indeed binds to this responsive element associating with *CDK2* expression and centrosome amplification. RT-PCR and Western blotting results revealed that both mRNA and protein levels of *CDK2* were significantly higher in tumor tissues than those in paired adjacent normal laryngeal tissues.

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Introduction

Centrosomes duplication is a crucial event when cell divided, which is indispensable for the formation of the bipolar mitotic spindle and plays an essential role in the maintenance of chromosomal stability (1). Recent developments have addressed the impact of numerical centrosomal amplification on the cellular changes associated with tumorigenesis and chromosomal segregational defects in the cancer cell (2). Centrosome abnormalities, in particular amplification, have been found in many types of tumors, such as non-small cell lung cancer (3-5). It has been previously proposed that centrosome amplification can drive chromosomal instability (CIN), a major source of aneuploidy (6-9), thereby, has considerable implications not only for tumor initiation but also for tumor cell evolution (10,11). Centrosomes amplification have been observed in human laryngeal squamous cell cancer cell line Hep-2 (12), but the detailed mechanisms yet to be investigated.

The transcription factor NF-κB is a family of closely related homo- or hetero-dimers sharing a Rel homology domain that mediates dimerization, interaction with IκB proteins (13). It regulates inducible gene expression in various physiological settings which is important in the regulation of immune response, cell apoptosis, cell cycle progression, proliferation and oncogenesis (14). Constitutive activation of the NF-κB has been documented to be involved in the pathogenesis of many human malignancies, such as prostate cancer, Mantle cell lymphoma (15,16). Inhibition of constitutive NF-κB signaling in each of these cancer types induces apoptosis. In unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by their association with inhibitors of NF-κB (IκBs), which prevent nuclear translocation of NF-κB dimers (17). During the past few years, tremendous progress has been achieved in our understanding on how intracellular signaling pathways are transmitted in either a linear or a network manner leading to the activation of NF-κB and subsequent cell growth control factors (14). It has been reported that the positive expression rates of NF-κB in patients with LSCC was significantly higher than this in the adjacent normal tissue and NF-κB

has been a useful prognostic marker of LSCC (18). Starska *et al* reported that elevated NF-κB expression has been observed in laryngeal carcinoma tumor cells by immunohistochemistry among 45 patients (19). However, the effect of NF-κB on centrosome amplification which is involved in cell proliferation and oncogenesis has yet to be studied. Elucidation of the relationships between NF-κB activation and centrosome amplification will be important in developing new strategies for the treatment of various human diseases, such as cancer. *CDK2* is an important factor in regulating cell proliferation and coordinating cell cycle with centrosome cycle, we analyzed a 1556-bp fragment of the *CDK2* promoter with TRANSFAC-TESS. This analysis revealed that there were four potential NF-κB elements in *CDK2* promoter region, thus *CDK2* maybe play a role in NF-κB induced centrosome amplification.

Laryngeal carcinoma is one of the most common tumors occurring in the head and neck regions, and has a high prevalence in northeast China. Pathologically, about 93-99% percent of laryngeal carcinomas are of squamous cell type (12). These previous studies suggested that centrosome amplification may be one of the key mechanisms underlying laryngeal carcinogenesis. In this study, the effect of NF-κB on centrosome amplification was detected. Furthermore, *CDK2* expression regulated by NF-κB was explored.

Materials and methods

Tissue samples. Resected human laryngeal carcinoma and adjacent normal tissues, obtained with informed consent, from the same patient were frozen with liquid nitrogen immediately after operations, and stored at -80˚C, all 76 cases of tumors were from patients of northeast China origin and designated as squamous cell carcinoma by pathological diagnosis.

Cell culture and treatment with BAY11-7082. A human laryngeal squamous cell cancer cell line Hep-2 and human embryonic kidney cell line (HEK293) was cultured in RPMI-1640 supplemented with 10% fetal calf serum. Hep-2 was plated in 6-well plates the day before treated with BAY11-7082 or DMSO as control. Cells were harvested to detect *CDK2* expression levels at 0, 3, 6, 12 and 24 h after treatment with BAY11-7082 or DMSO.

Transfection. HEK 293 cells were grown on 6-well plates and transfected with 4 μg of pEGFP-C1-NF-κB plasmid by Lipofectamine 2000 (Invitrogen) according to specifications. Forty-eight hours after transfection cells were collected, Western blotting were performed to detect the *CDK2* expression.

MTT assay. Hep-2 cells were grown on 96-well plates and allowed to adhere overnight. The cultures were washed and refreshed with a serial concentration of BAY11-7082 (20, 15, 10, 5, 2.5, 1 μmol/l). After 24-h incubation, 100 μl MTT was added into each well and incubated for 4 h. At the end of the treatment the incubation medium was removed and the formazan crystals were dissolved in 150 μl of DMSO. MTT reduction was quantified by measuring the light absorbance of each well at 490 nm to evaluate Hep-2 cells proliferation. All experiments were performed in triplicate.

Analysis of centrosome amplification by immunofluorescence microscopy. Hep-2 cell line were cultured on slides and then fixed with ice cold methanol at -20˚C for 12-14 h. After fixation, they were postfixed in ice-cold acetone for 6 min, rinsed for 5 min in phosphate-buffer saline (PBS, pH 7.0) twice, blocked with 5% BSA in PBS at room temperature for 40 min. The primary antibody (monoclonal anti-γ-tubulin antibody derived from a mouse with GTU-88 hybridoma, Sigma, St. Louis, MO, USA) was diluted 1:250 in PBS at 37˚C for 2 h. Three 5 min washes were performed in PBS. The primary antibody was detected by incubation with secondary antibody, FITC-conjugated goat anti-mouse IgG (Sigma), diluted at 1:125 in PBS. Incubation was carried out at 37˚C for 45 min in a dark moist chamber. After that slides were washed three times for 10 min in PBS at room temperature. The nuclei were counterstained with 4,6-diamino-2-phenylindole (DAPI), five slides were selected from each line and numbers of centrosomes were counted in 200 cells under fluorescence microscope.

Whole-cell lysate preparation and immunoblotting. For sample preparation, 100 mg of tissue was taken from each sample and ground to a powdery preparation with liquid nitrogen. The sample then underwent a process of homogenizing for 10 sec in the presence of cell lysis buffer for Western and IP, ice-bath for 30 min, and centrifugation at 12,000 g for 30 min at 4˚C. Cells were solubilized with lysis buffer, after 30 min of incubation on ice, lysates were clarified by centrifugation (12,000 g) for 30 min at 4˚C and supernatants were collected. The samples concentration was determined by an ultraviolet spectrophotometer. Proteins (60 μg/lane) were separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels. Gel-resolved proteins were subsequently electrotransferred to nitrocellulose membranes, which were blocked with 1% BSA, incubated with various primary antibodies used at 1:2,000 dilutions, and developed with HRP-conjugated secondary antibodies (ZhongShan, China, 1:4000). The blot was treated with ECL reagent and exposed to film. The image was captured by transmission scanner with the internal control of the β-actin protein level, and relative quantitative analysis was carried out based on the photo density ratio.

Plasmid constructions. A series of truncated *CDK2* promoter fragments was amplified from Hep-2 genomic DNA using Pfu turbo (Stratagene, CA, USA) according to the manufacturer's instructions, spanning -1442/+114, -1334/+114, -1261/+114, -732/+114 and -199/+114 (+1 indicates the transcription start site), using different forward primers and the same reverse primer as follows: -1442- F: 5'-GAGCTCGATACCCCATTCTT TCTCCAC-3'. -1334- F: 5'-GAGCTCCCCCAGAGCCCTTTC ATG-3'.-1261-F:5'-GAGCTCGGGCTGTGTTCACCCGTA-3'. -732- F: 5'-GAGCTCACTGGGGGGACTGGGATA-3'. -199-F: 5'-GAGCTCTAGAGACATAGGTAGGAAACTTGG-3'. +114-R: 5'-AAGCTTCGGGATGGAACGCAGTAT-3'.

The fragments were cloned into pMD18-T vectors, subcloned into the unique *Sac*I/*Hin*dIII sites of pGL3-Basic vector (E1751, Promega, USA) to generate pGL3-CDK2-Luc plasmid, and named pGL3-1442, pGL3-1334, pGL3-1261, pGL3-732 and pGL3-199.

Overlap extension-PCR was performed to generate mutations in the *CDK2* promoter, putative NF-κB binding site nos. I, II

and III were mutated to generate MutI, MutII and MutIII from the parental pGL3-1442 plasmid. Therefore three nucleotides in the putative NF-κB-binding sites were changed, respectively. The mutagenic primer pairs are as follows: MutI F: 5'-cctcagagc agcacggcagaatccagaccctctcacc-3'; R: 5'-ggtgagagggtctggattctgc cgtgctgctctgagg-3'. MutII F: 5'-gtgatgctcagctgagtcgcgtctgccta tctctccag-3'; R: 5'-ctggagagataggcagacgcgactcagctgagcatcac-3'. MutIII F: 5'-gagattattttcaggcaaagttcgaaatctccatgtaggg-3'; R: 5'-ccctacatggagatttcgaactttgcctgaaaataatctc-3'. Mutant clones were sequenced to verify the mutations.

Luciferase reporter gene assay. Hep-2 was plated in 24-well plates the day before transfection and grown to ~70% confluence. Cells were then transiently co-transfected with a DNA mix containing 1 μg of the series of deleted *CDK2*-luciferase constructs, 0.1 μg of pRL-TK plasmid (Promega) as the internal control and 0.25 μg of either the NF-κB expression plasmid pEGFP-NF-κB or control vector pEGFP-C1 by Lipofectamine 2000 (Invitrogen), The Dual-Glo™ Luciferase Assay (Promega) was used to determine firefly and renilla luciferase activity 36 h after transfection. Furthermore, to test the effects of mutation of NF-κB binding sites in the *CDK2* promoter on the ability of NF-κB to Trans-activate *CDK2*, equimolar amounts of pGL3-1442 (wt), MutI, MutII and MutIII were transfected using Lipofectamine 2000 into Hep-2 cells.

Electrophoretic mobility shift assays (EMSA). Nucleoprotein was extracted from Hep-2 using a cytoplasmic and nuclear protein extract kit (Activ Motif, USA) according to the instructions. Oligonucleotides and their complementary strands were used to evaluate binding of NF-κB to the *CDK2* promoter NF-κB binding site (5'-aaatcccaaa-3'). The double-strand wild-type DNA (aggcaaaatcccaaatctccat) was labeled with biotin according to standard protocols (Pierce, USA) and mutated sequence (aggcaaagttcgaaatctccat) was synthesized. A 100-fold excess of unlabeled probe was used as a specific competitor. The DNA-binding ability of the different proteins was monitored by EMSA on a 6% non-denaturing polyacrylamide gel. DNA binding bands were detected using a chemiluminescence system (Pierce, USA).

Chromatin immunoprecipitation. Hep-2 cells were cross-linked with 1% formaldehyde for 10 min at room temperature, followed by addition of glycine to 0.125 M and continued incubation for 5 min. Cells were lysed in hypotonic buffer, and nuclear pellets were collected by centrifugation and resuspended in nuclear lysis buffer. Solubilized chromatin was sheared by sonication. Chromatin was incubated with goat anti-NF-κB antibody (cat.1546-1; Epitomics) or goat IgG at 4˚C overnight. Complexes were recovered by incubation with A/G Plus-agarose beads at 4˚C for 2 h. Beads were collected by centrifugation and washed thoroughly. Complexes were eluted by incubation with 150 μl immunoprecipitation elution buffer (1% SDS and 0.1% $NaHCO_3$). NaCl was then added at a final concentration of 0.3 M, and samples were heated at 65˚C overnight to reverse cross-linking, followed by phenol-chloroform extraction and ethanol precipitation. DNA was resuspended in 30 μl TE, and PCR was performed using primers corresponding to the regions -1,016 to -999 (5'-caggcaaaatcccaaatc-3') and -894 to -911 (5'-tctggtttctgtcctcct-3'), which span conserved NF-κB binding site no. 3 in the human *CDK2* promoter at -1,010 to -1,001 relative to transcription start site at +1. For input DNA, soluble chromatin not incubated with antibodies was subjected to PCR. A PCR control was performed using primers corresponding to the other regions of *CDK2* promoter that no putative NF-κB binding site.

Semi-quantitative RT-PCR analysis. Total RNA was isolated by TRIzol reagent according to the instructions, and cDNA was reversibly transcribed from the isolated mRNA using an AMV RNA PCR Kit (Takara) in line with the standard operational protocol. Primer sequences for *CDK2* primers were: F: 5'-AGC CAGAAACAAGTTGACGG-3' R: 5'-TGATGAGGGGAAGA GGAATG-3', and the amplified fragment was 251 bp in length. β-actin primers were: F: 5'-CCAGATCATGTTTGAGACCT-3' R: 5'-TTGAAGGTAGTTTCGTGGAT-3', and the amplified fragment was 480 bp.

The PCR reaction was performed in a 25-μl reaction system, starting with denaturation at 94˚C for 3 min, then 30 cycles of denaturation at 94˚C for 30 sec, annealing at 58˚C for 30 sec, extension at 72˚C for 45 sec, followed by an extra extension at 72˚C for 10 min. The PCR product was examined by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The gel image scanning analytical system was able to provide the photo density of the electrophoresis band of *CDK2* and β-actin as the internal control. Relative quantitative analysis was carried out based on the photo density ratio (*CDK2*/β-actin).

Data analysis. Statistical analysis was performed using SPSS. All data were expressed as the mean ± standard deviation (SD). Statistical comparisons were carried out using one-way analysis of variance (ANOVA), randomized block analysis of variance, and the Students-Newman-Keuls (SNK) method was employed for intergroup comparison. Associations between categorical variables were verified by χ^2 test. Statistical significance was determined as $p<0.05$.

Results

BAY11-7082 inhibited Hep-2 cell proliferation and cenreosome amplification. The effect of BAY11-7082 on Hep-2 cell proliferation was determined by MTT assay. As shown in Fig. 1A, different concentrations of BAY11-7082 exerted different effects on Hep-2 proliferation capacity. BAY11-7082 was found to have marked inhibitory on Hep-2 cells proliferation, IC_{50} value is 5 μmol/l.

Among the 5 samples of Hep-2 cell line treated with BAY11-7082, the number of centrosomes in a single cell varied from 1-3, ~2% of cell were found to have more than two centrosomes. While among the Hep-2 cell line samples, apparent centrosomal amplification was found in ~8% of cells, with the numbers of centrosome varying from 1-7 (Fig. 1B). By χ^2 test, samples from Hep-2 cell line had significantly more aberrations ($p<0.05$) (Fig. 1C).

Transcriptional activation of CDK2 gene by NF-κB. To determine weather the up-regulation of *CDK2* correlates with p65, BAY11-7082 was used as an inhibitor that blocks NF-κB degradation, *CDK2* levels were detected by Western blotting at 0, 3, 6, 12 and 24 h after treatment (Fig. 2A), the results show that BAY11-7082 inhibited *CDK2* expression significantly in compare with

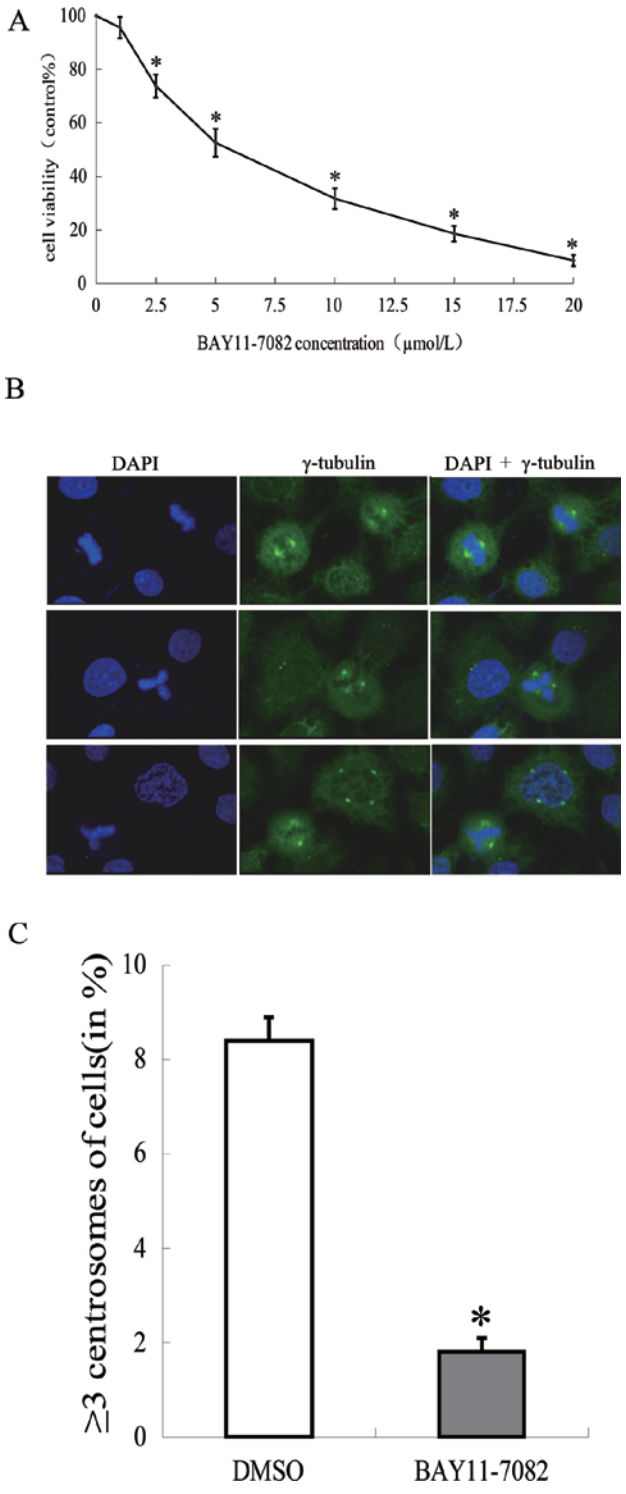

Figure 1. The effect of BAY11-7082 on cell proliferation and centrosome amplification. (A) MTT assay. Hep-2 cells were exposed to different concentration of BAY11-7082 for 24 h, MTT assay was performed to evaluate the effect of BAY11-7082 on Hep-2 cells proliferation. The cell viability was calculated after detection of absorbance in 490 nm. Mean ± SD (n=3). *p<0.05 vs. the levels of control. (B) Hep-2 cells containing different number of centrosomes (counterstained with DAPI). (C) BAY11-7082 inhibited centrosome amplification in Hep-2 cells. *p<0.05 vs. the levels of control.

DMSO control. Furthermore, HEK 293 cells were transfected with a p65 expression vector. Western blotting was performed to detect *CDK2* protein levels 48 h after transfection and over-expression of p65 effectively increased the levels of *CDK2* 1.6-fold (Fig. 2B). To elucidate the role of NF-κB in the transcriptional activation of *CDK2*, we constructed a luciferase vector containing ~1.5 kb *CDK2* promoter region and transfected the promoter constructs into Hep-2 cells. Cells were co-transfected with the p65 expression vector or empty vector. Luciferase assays indicate that expression of p65 effectively increases the activity of the *CDK2* promoter 3-fold (Fig. 2C). Moreover, cells were treated or untreated with 5 μM BAY11-7082 after transfected the promoter constructs. Luciferase activity decreased dignificantly compared to DMSO control (Fig. 2D). These results suggest that NF-κB signaling plays a role in up-regulation of *CDK2*.

Identification of NF-κB responsive element within CDK2 promoter. The sequence of the *CDK2* promoter starting -1442 bp before the transcriptional start site was analyzed with the TFSEARCH tool. Four potential NF-κB binding sites were predicted in *CDK2* promoter. A site located between -1398 and -1390 bp from of the transcription start site was denoted site I. Site II was located at -1300/-1294, site III was located at -1011/1001 and site IV located at -216/-207 (Fig. 3A). To evaluate the importance of these sites and the mechanisms controlling transcriptional regulation of *CDK2* in Hep-2 cells a series of deletion constructs were generated (Fig. 3B). Cotransfections in Hep-2 cells of these *CDK2*-luciferase constructs and the NF-κB expression plasmid pEGFP-C1-NF-κB demonstrated trans-activation of the *CDK2* promoter by NF-κB compared with the control vector pEGFP-C1 (Fig. 3C). Greatest activation of the *CDK2* promoter by NF-κB was seen with -1442-*CDK2*-Luc by 3-fold. Trans-activation of *CDK2* by NF-κB declines progressively with deletion of the *CDK2* promoter spanning from -1442 to -732 in Hep-2 cells, suggesting the presence of functional NF-κB binding sites within the deleted regions. But deletion of sequence from -731 to -200 resulted in no significant change in luciferase activity suggesting that no important NF-κB regulatory elements reside in this region.

Furthermore, to illuminate the importance of these potential NF-κB binding sites, we next conducted site-directed mutagenesis to disrupt the NF-κB binding sites in CDK2 promoter constructs (Fig. 4A). Promoter activity of mutated constructs was determined in Hep-2 cells and compared with that of the wild type pgl3-1442 construct. Single mutation of the NF-κB sites I or II produced no significant change in promoter activity. Single mutation of the NF-κB site III (construct mutIII) however resulted in 76±5% ($p<0.01$) reductions (Fig. 4B). From the above mutation and previous deletion analysis, we defined the region spanning -1010 to -1001 as the single NF-κB binding site in the *CDK2* promoter. This suggests that NF-κB site III is important for *CDK2* upregulation in response to p65.

P65 binds to the 5'-promoter region of CDK2. Finally, in order to confirm whether NF-κB indeed binds to the 5'-promoter region of *CDK2* gene, we performed EMSA and ChIP assays. In the former analysis, we obtained a clear band with a hot probe containing the NF-κB element derived from the *CDK2* promoter (Fig. 5A). This band was completely eliminated with a cold probe, but not with mutant NF-κB sequence, confirming the specificity. In ChIP assay, we also found a PCR product of an appropriate size using *CDK2* gene 5'-promoter-derived

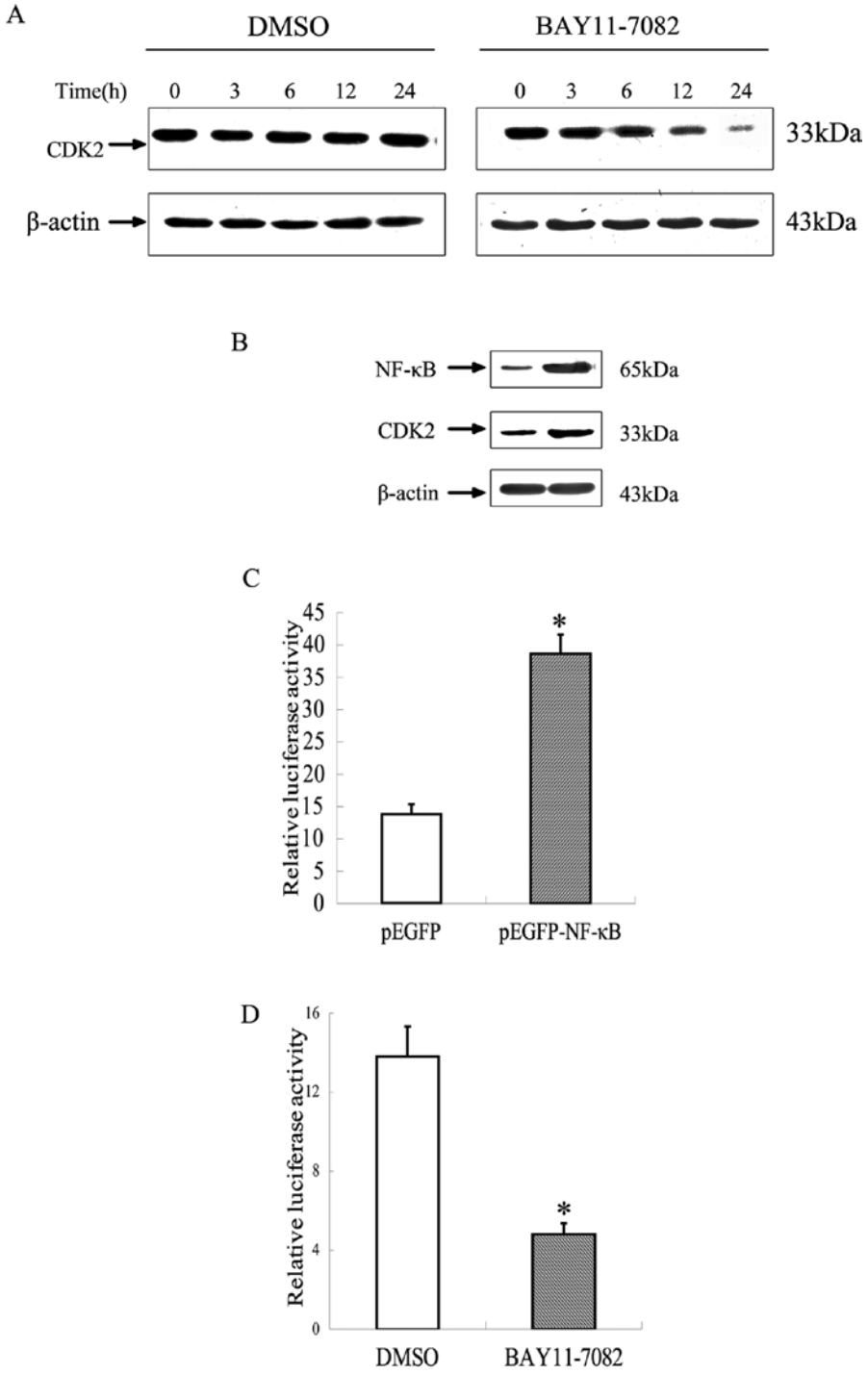

Figure 2. p65 plays an important role in the induction of *CDK2* expression. (A) BAY11-7082 inhabited *CDK2* expression, Hep-2 cells were treated with 5 μM BAY11-7082 or DMSO as a control and harvested at 0, 3, 6, 12 and 24 h after treatment. *CDK2* levels were detected by Western blotting. (B) p65 over-expression increases the levels of *CDK2*. Twenty-four hours after transfected cells were lysed, *CDK2* and p65 levels were detected by Western blotting. (C) p65 increases transcriptional activity of *CDK2* promoter. Hep-2 cells were transfected with the luciferase vector pGL3 containing the *CDK2* promoter region or empty pGL3 vector and were co-transfected with an expression vector for p65 or empty vector, luciferase assays were performed after 24 h. Data represent the means ± SD (n=3). $^*p<0.05$ vs. the levels of control. (D) Hep-2 cells were transfected with the luciferase vector pGL3 containing the *CDK2* promoter region or empty pGL3 vector and treated with or without 5 μM of the inhibitor BAY11-7082. Cells were lysed after 24 h and luciferase assays were performed. Data represent the means ± SD (n=3). $^*p<0.05$ vs. the levels of control.

primer sets harboring the NF-κB element (-1010 to -1001), but no PCR product was obtained with IgG and negative control primer sets (Fig. 5B).

Expression of the CDK2 gene in laryngeal squamous cell cancer (LSCC). With the purpose of revealing the clinical significance of *CDK2* expression in laryngeal squamous cell cancer, RT-PCR and Western blotting were performed to assess the expression of *CDK2* among the 76 cases of laryngeal carcinoma analyzed. There were 50 (66%, $p<0.05$) whose mRNA expression level of *CDK2* gene was higher in tumor tissues than in pair-control normal tissues (Fig. 6A and Table I), 48 (63%, $p<0.05$, χ^2 test) in

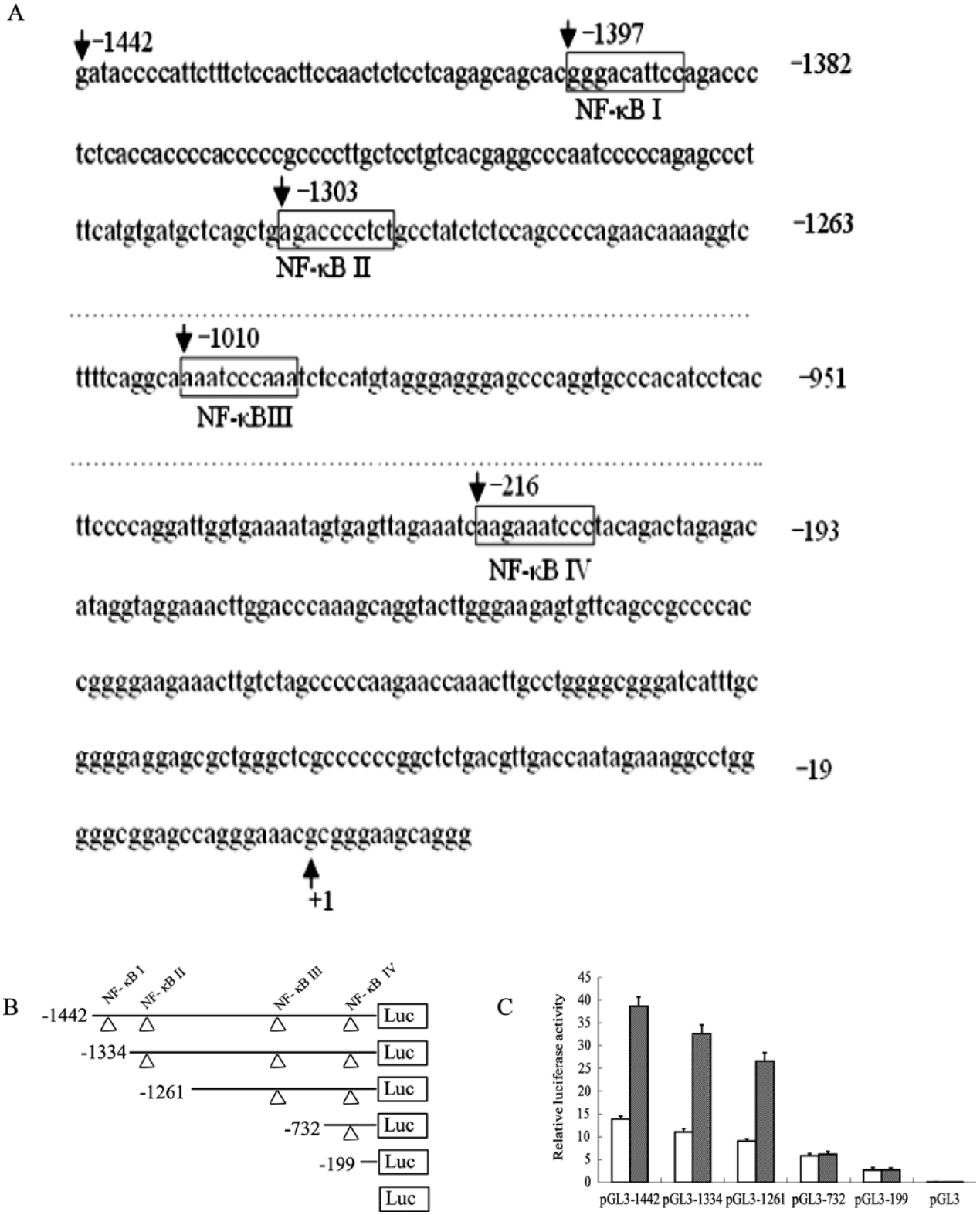

Figure 3. Mapping of NF-κB responsive element. (A) Putative cis-acting elements within *CDK2* promoter by TRANSFAC-TESS (B) Promoter-reporter construct containing deleted NF-κB -binding sites. (C) Luciferase analysis of deleted constructs. Cells were transfected with plasmids containing a series of deletion mutant *CDK2* gene promoters (-1442, -1334, -1261, -732 and -199 bp, and promoterless constructs; +1 designates the transcription start site) and NF-κB expression vector [CDK2-luc (μg): NF-κB (μg) = 1:1], demonstrate trans-activation of the *CDK2* promoter by NF-κB compared with the empty vector pEGFPC1. Firefly luciferase activity was measured 24 h after transfection and normalized to Renilla luciferase activity. Data represent the means ± SD (n=3). *p<0.05 vs. the levels of control.

protein levels (Fig. 6B and Table I). The results of the statistical analysis also indicated that the high levels of *CDK2* were not associated with laryngeal squamous cell cancer patient gender, age and TNM stage (data not shown).

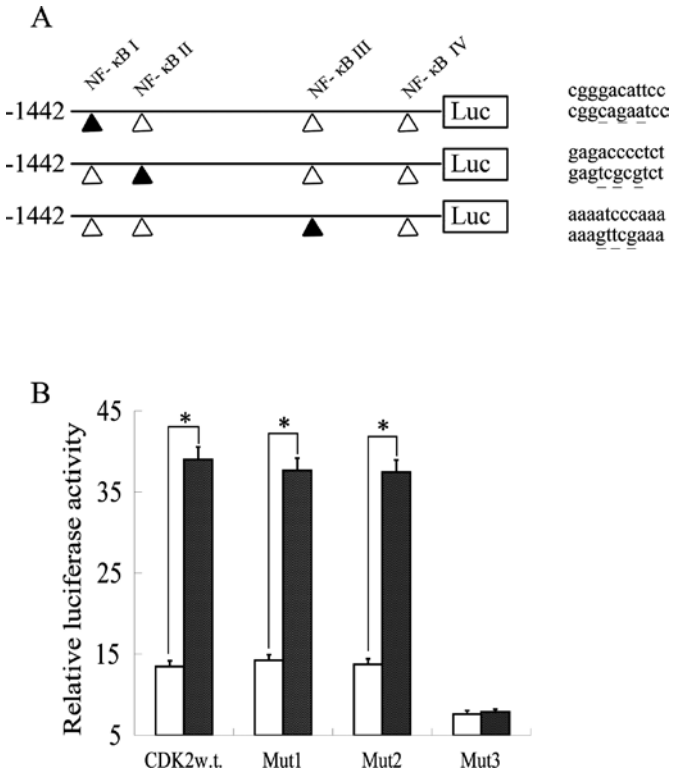

Figure 4. NF-κB site III is crucial for *CDK2* up-regulation in response to p65. (A) Site directed mutagenesis was performed on the pGL3-1442/+114 plasmid. Three different plasmids were generated: pGL-Mut I containing point mutation of site I, pGL-Mut2 II containing a point mutation of site II, and pGL-Mut III containing point mutation of critical sites for NF-κB binding in site III. The wild-type and mutated sequences of these sites are listed. (B) Mutation analysis of NF-κB binding. Hep-2 cells were transfected with luciferase vector containing one of the Mut I, Mut II, or Mut III mutations; where indicated cells were co-transfected with a p65 expression vector. After 24 h cells were lysed and luciferase assays were performed. Data represent the means ± SD (n=3). *p<0.05 compared with -1442-CDK2-Luc.

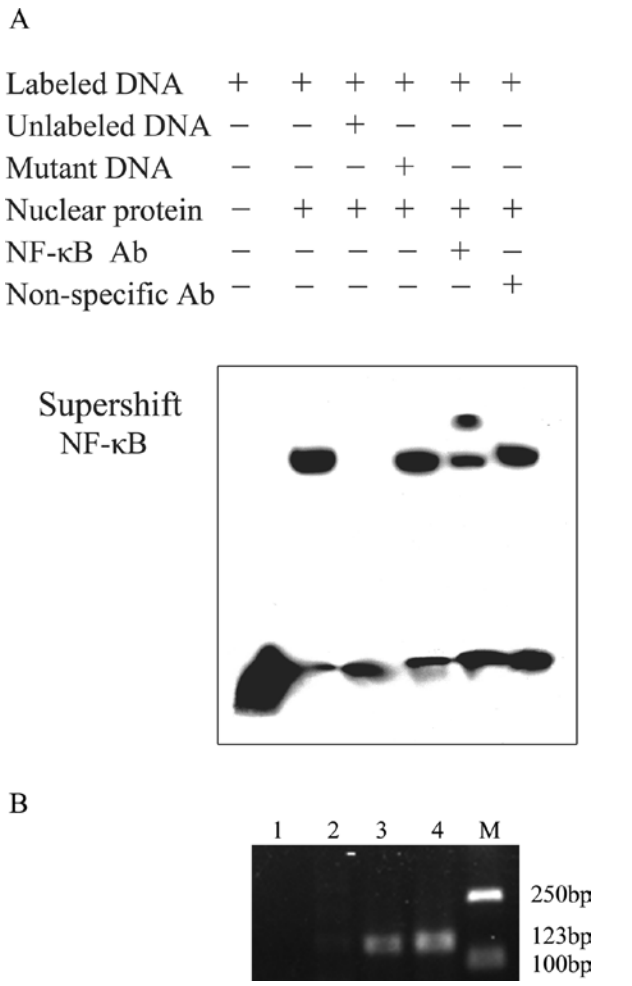

Figure 5. Binding of NF-κB to the *CDK2* promoter *in vitro* and *in vivo*. (A) EMSA assays were performed using a probe containing NF-κB site III sequence and mutant NF-κB site III sequence. In addition we added antibody against p65 and antibody against STK15 as a non-specific Ab (ctrl. Ab.). Lane 1, labeled probe without nuclear extract; lane 2, labeled probe incubated with nuclear extract; lane 3, competition experiments performed with a 100-fold molar excess of unlabeled probe sequence; lane 4, 100-fold molar excess of unlabeled mutant sequence did not compete the labeled probe from complex; lane 5, supershift analysis of NF-κB bound to the probe performed using anti-NF-κB antibody; lane 6, no supershift appeared with ctrl. Ab. (B) Analysis of NF-κB binding to the *CDK2* promoter *in vivo* by ChIP assay. Lysates of Hep-2 cells were immunoprecipitated with NF-κB antibody or normal rabbit IgG. Subsequently, a DNA segment of *CDK2* promoter containing NF-κB site III was amplified by PCR. Lysates without immunoprecipitation were used as the control for DNA input in each sample. 1, negative PCR control; 2, control IgG; 3, anti-P65 4, input M, marker.

Discussion

Many factors can initiate tumorigenesis, more and more evidence implicated centrosome amplification in the origin of chromosomal instability during tumor development. Chromosomal instability (CIN) correlates with the presence of extra centrosomes and it is a common feature of tumor cells (20). As the center for microtubule organizing, centrosomes ensure the fidelity of chromosomal segregation into two daughter cells and the stability of genome by forming the bipolar spindle during mitosis. Whereas, centrosomal amplification may promote chromosomal instability (CIN) and so tumorigenesis. In support of this hypothesis, it has recently been shown that centrosome amplification can initiate tumorigenesis in flies (6). Recent work in *Drosophila* also suggests that centrosome defects in asymmetrically dividing cells can induce tumors at a higher frequency than other conditions known to cause genomic instability. Previous study suggested that inducing genetic instability in mice can increase the rates of tumor formation in some, but not all tissues (21). One mechanism of centrosome amplification mediated CIN is the clustering of the centrosomes into two centrosomal groups during mitosis to allow the formation of a bipolar spindle (4). Missegregation not only distorts the number of chromosomes in the daughter cells but also promotes or diminishes the expression of genes critical for cell viability and growth (1). The conventional wisdom has been that aneuploidy is a late event in cancer development, but recent evidence has suggested that aneuploidy is an early change for malignancy (22). Some studies have shown that centrosome may play a role in the early stage of tumorigenesis through chromosomal instability. As a common type of abnormality, centrosomal amplification has been found in many kinds of tumors including those of breast, adrenocortica glands, and acute myeloid leukemia. We have discovered a higher proportion of cells harboring amplified cengtrosomes in Hep-2 cell line, which may be a mechanism of Laryngeal carcinoma tumorigenesis.

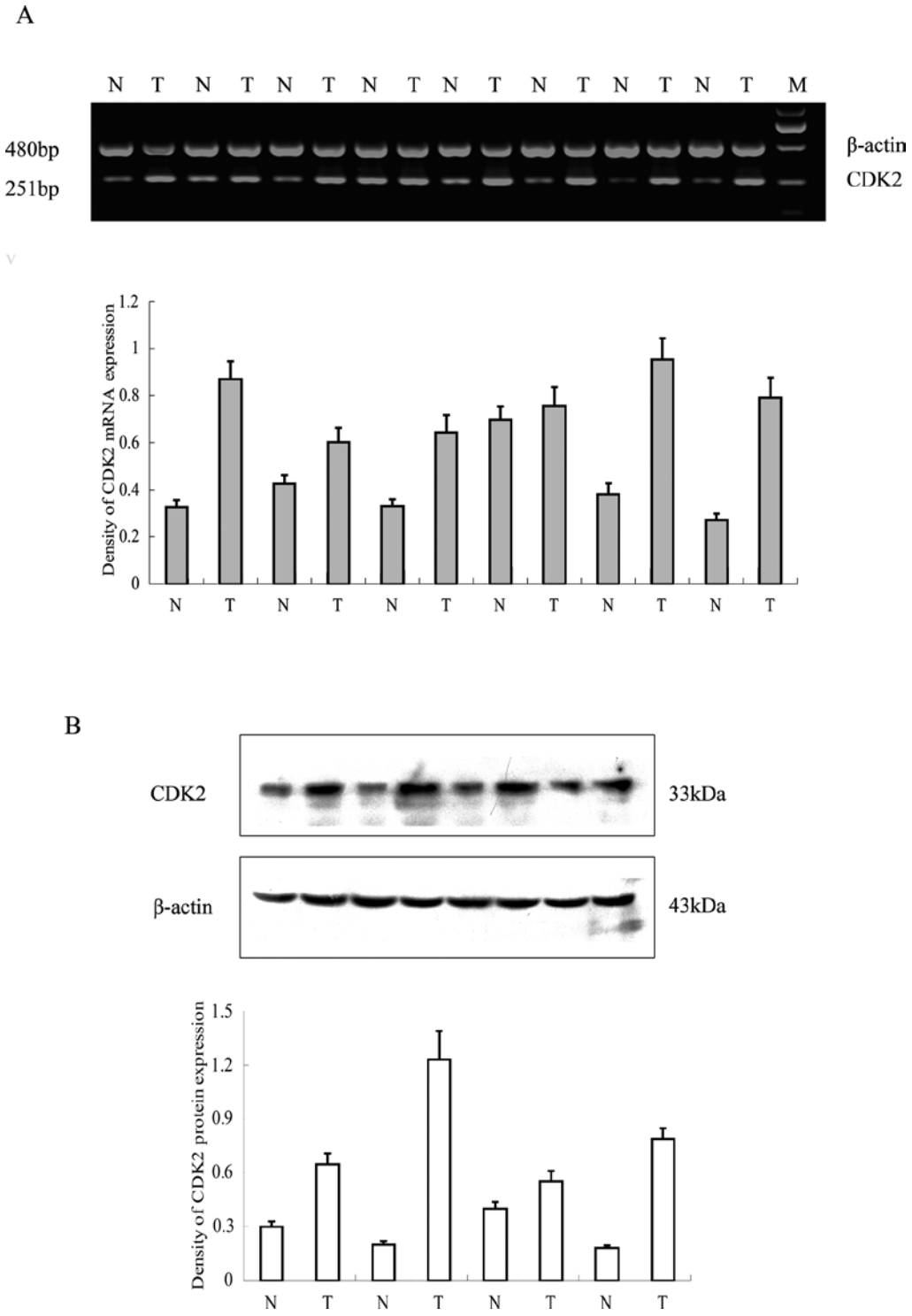

Figure 6. Overexpression of *CDK2* gene in tumor tissues (N, normal tissues; T, tumor tissues). (A) *CDK2* mRNA expression level is higher in tumor than in normal tissues. (B) *CDK2* protein expression level is higher in tumor than in normal tissues.

Table I. Analysis of *CDK2* mRNA and protein levels in different tissues (n=76).

Tissues	*CDK2* mRNA	*CDK2* protein
Normal	0.384±0.108	0.235±0.065
Tumor	0.786±0.253	0.722±0.162
p-value	<0.05	<0.05

Abnormal centrosome amplification occurs frequently during cellular transformation (23,24), hence, factors contributing to the regulation of centriole duplication are likely to play a role in cancer development (25,26). Furthermore, the deregulation of some regulators of centrosome duplication has already been shown to contribute to tumor formation. Many proteins related to centrosome replication have been identified, including *CDK2, NDR1, Bmi1* and *Plk2*. Meanwhile, the cell must not only control centrosome copy number but also coordinate the

events of centrosome reproduction with nuclear events in the cell cycle (27). *CDK2* (cyclin-dependent kinase 2), a G1/S promoting kinase, is a key regulator which coordinate of centrosome duplication with the cell cycle. In late G1, *CDK2* relocates into cyclin A-containing complexes that are responsible for coordinating the events of S-phase (28). Previous studies have also suggested that *CDK2* is required for centrosome duplication in mammalian cells and revealed that it depends on *CDK2* for the coordination of centrosome duplication with the cell cycle (29); data show that *CDK2* has a high level in many kinds of cancer cells (30,31). Furthermore, *CDK2* is required for oncogene-induced centrosome overduplication (32). Suppression of *CDK2* expression induces cell cycle arrest and cell proliferation inhibition in human cancer (33). Another research group reported that improved attachment to culture substratum inhibition of *CDK2* diminished the frequency of multicentrosomal mitoses (34). In this study, we find that *CDK2* displays a higher expression in the LSCC tissue than that in the adjacent normal mucosa. Analysis of the relationship between the *CDK2* expression of LSCC tissue and the cetrosomes amplification implies that *CDK2* might play a role in the centrosomes abnormality of LSCC.

Most researches are focused on searching for inhibitors of *CDK2* and regulating the activity of *CDK2*/cyclin E (31), the mechanisms leading to *CDK2* abnormal expression and so centrosomal amplification are not defined. The understanding of the promoter regions essential for gene transcription is very important to study the molecular mechanisms that pertain to diseases and a prerequisite for targeted intervention in gene expression (35). There is emerging evidence that NF-κB is a critical factor in tumor development, growth and metastasis (13). The results of our inhibitor examination indicated that BAY11-7082 inhibited *CDK2* expression significantly. Overexpression of NF-κB can increase *CDK2* protein level. To characterize the regulation of *CDK2* gene expression, specifically at the level of transcription by NF-κB, we cloned and identified the human *CDK2* promoter region spanning from -1442 to +114 relative to the transcription start site. There were four potential NF-κB binding motifs located in the promoter region. We found that removal of the part of the *CDK2* promoter containing three NF-κB binding sites decreases promoter activity remarkably, suggesting that the regulatory elements in this region bind transcriptional activators. Site mutation of the NF-κB binding site III displays similar effect of promoter activity as removal of *CDK2* promoter containing three NF-κB binding sites. EMSA using nuclear extracts from human Hep-2 lines demonstrated specific binding of NF-κB to sequences representing the third site (-1011/1001). However, results obtained from these constructs in vitro systems may not necessarily reflect the regulation and function of the endogenous gene. In this study, we corroborated the EMSA and transient transfection data using ChIP analysis. Data from these experiments demonstrated that NF-κB was specifically associated with the *CDK2* promoter in laryngeal squamous cancer cells (Fig. 5). NF-κB binds to the *CDK2* promoter, leading to an enhancement of transcriptional activity. Taken together, our results hypothesize that NF-κB interacts with the *CDK2* promoter and acts as an initiator in centrosome amplification. Our result suggested that there was a NF-κB responsive element in *CDK2* gene promoter region and indicated that NF-κB regulated *CDK2* expression and centrosome amplification, so elucidated how NF-κB promote cell proliferation coordinating with centrosome duplication.

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