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THE NOVEL PROTEIN TSR2 INHIBITS THE TRANSCRIPTIONAL ACTIVITY OF NUCLEAR FACTOR-κB AND INDUCES APOPTOSIS

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The nuclear factor-κB (NF-κB) pathway is involved in a variety of cellular functions, including cell proliferation, differentiation, development, oncogenesis, and apoptosis. In this study, we report on cloning and characterization of the human TSR2 (also known as 20S rRNA accumulation homolog), a protein containing a WGG motif, which has no known specific function, although this protein is conserved during evolution across different species. The cDNA sequence contains a 576 bp open reading frame, encoding a 191 amino acid protein with a predicted molecular mass of 20.9 kDa. Northern blot analysis revealed broad *TSR2* mRNA expression in human tissues. Overexpression of *TSR2* in human epidermal HEp-2 cells inhibited the transcriptional activity of NF-κB, with or without tumor necrosis factor α stimulus, and induced HEp-2 cell apoptosis. This data for the first time suggests that TSR2 is involved in the NF-κB signaling pathway and may regulate apoptosis.

***Keywords*: TSR2, nuclear factor-kappaB, apoptosis, laryngeal cancer.**

INTRODUCTION

Nuclear factor-κB (NF-κB) is a transcription factor that plays a critical role in many cellular processes, including embryonic and neuronal development, cell proliferation, apoptosis, inflammation, and immune response to infections [1–5]. Disregulation of NF-κB signaling is associated with inflammatory diseases and certain cancers [6–8]. Experimental *in vitro* and *in vivo* studies demonstrated that NF-κB suppresses the development of carcinogen-induced tumors, inhibits the growth of cancer cells, and induces apoptosis by altering gene expression, which is critical for the control of carcinogenesis and cancer cell survival. NF-κB inhibition can induce apoptosis [9–11]. Therefore, agents or genes that suppress NF-κB may be useful in the prevention and treatment of cancer [9, 12, 13].

The WGG motif is a conserved sequence of a family of proteins described as pre-rRNA-processing proteins. In this study, we amplified the cDNA for TSR2 (also known as 20S rRNA accumulation homolog), a WGG motif-containing protein with no known function. The reverse transcription PCR (RT-PCR) assay and real-time PCR analysis showed that *TSR2* expression was weak in HEp-2 cells and therefore, we selected the HEp-2 cell line for further studies, including those on the effects of TSR2 on the activity of transcription factors. Tumor necrosis factor (TNF)-induced activation of NF-κB is well established in several cell lines, including HEp-2 cells [14, 15]. This data may provide new clues for clinical application of TSR2 in laryngeal cancer.

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EXPERIMENTAL

cDNA cloning and expression vector construction. Sequence identity and open reading frame (ORF) prediction were performed using the human RefSeq database (http://www.ncbi.nlm.nih.gov/projects/RefSeq/) and expressed sequence tag (EST) database in GenBank. The full-length cDNA of human TSR2 (GenBank Acc. no. NM_058163) was amplified from the mixed human cDNA library ("Clontech") using the forward primer P1 (5'-taa tgg cgg gcg ctg ca-3') and reverse primer P2 (5'-ctc tca ttt ttt tct ccg gac-3'). The purified PCR product was ligated into a T-vector ("Promega") and sequenced. Then the insert fragment was excised with EcoRI and subcloned into the mammalian expression vector pcDNA3.1/myc-His(-)B ("Invitrogen") to construct the plasmid pcDNA-TSR2. The full-length coding region of *TSR2* cDNA was also cloned into pEGFP-N1 ("Clontech") to induce the expression of a C-terminal GFP fusion protein. Cells were analyzed with an Olympus IX 71 fluorescent microscope and photographs were captured using an Alta U2 digital camera ("Apogee Instruments Inc.", USA).

Northern blot and RT-PCRassay. *TSR2* cDNA labeled with fluorescein using the Gene Images Random Prime Labeling Kit ("Amersham Biosci.") according to the manufacturer's protocol was used as a probe. Total RNA was extracted from human tissues using the TRIzol reagent ("Invitrogen"). Samples (20 μg from each tissue specimen) were separated by electrophoresis and transferred onto a nylon membrane ("Amersham Biosci."), which was subsequently hybridized with the probe at 65°C overnight. After washing with the SSC buffer, the

membrane was incubated with anti-fluorescein-alkaline phosphatase (AP) conjugate and images were developed using a Gene Images CDP-Star Detection Module ("Amersham Biosci."). RT-PCR was performed on a ThermoScript RT-PCR System ("Invitrogen") using the P1 and P2 primers, with GAPDH as a control. To quantify the human *TSR2* expression levels in various cell lines, quantitative real-time PCR assay was performed using the ABI Sequence Detection System ("Applied Biosystems") with specific primers for *TSR2* (P1/P2) and β-actin (P3: 5'-cag cac aat gaa gat caa gat ca-3'; P4: 5'-cgg act cgt cat act cct gc-3'). PCR reactions were performed according to the manufacturer's instructions using the SYBR Green PCR Master Mix Kit under the following conditions: initial heat denaturation at 95°C for 10 min, followed by 35 cycles of 15 s each at 95°C and a final 1 min cycle at 60°C. *TSR2* expression in the HEp-2 cell line was considered as the baseline. The experiments were repeated twice with consistent results.

Cell culture and transfection. HEp-2 cell lines were obtained from the American Type Culture Collection ("Manassas", VA, USA) and cultured in Minimum Essential Medium supplemented with 10% fetal bovine serum ("Hyclone"). All experiments were performed on growing cells during logarithmic growth. HEp-2 cells were transfected with Lipofectamine 2000 ("Invitrogen") according to the manufacturer's instructions.

Transient expression reporter gene assay. HEp-2 cells were co-transfected with reporter constructs for pRL-TK (Renilla control luciferase, "Promega"), pNF-κB-Luc (firefly luciferase, "Stratagene") and the *TSR2* expression vector spcDNA-TSR2. After 24 h, some of the cells were stimulated with TNF-α (10 ng/ml). Each treatment group had triplicate samples. After 6 h, the cells were lysed in a standard lysis buffer and the cell lysates were assayed for luciferase activities according to the "Stratagene" protocol. Firefly luciferase activities for experimental constructs were normalized for transfection efficiency using Renilla luciferase activity as an internal control. Relative luciferase activities from these experiments were expressed as fold luminescence over the activity of an empty vector under basal conditions. Each experiment was repeated at least three times.

Electrophoretic mobility shift assay (EMSA). 24 h after transfection, HEp-2 cells were treated with TNF-α (10 ng/ml) for 2 h. The treated cells were trypsinized, washed twice with PBS and pelleted by centrifugation. The nuclear protein extracts were prepared with the NEPER Nuclear and Cytoplasmic Extraction Reagents ("Pierce") according to the provided protocols. The oligonucleotides for the NF-κB consensus sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and the mutant NF-κB (5'-AGT TGA GGC GAC TTT CCC AGG C-3') were obtained from Santa Cruz Biotechnology ("Santa Cruz") and were end-labeled with biotin. Nuclear extracts (5 μg) were incubated with labeled DNA probes (50 fmol) for 30 min at room temperature in a total of 20 μl of the binding reaction mixture containing ×10 binding buffer, 50% glycerol, 1 μg/μl poly(dI-dC), as directed by the protocol from the LightShift Chemiluminescent EMSA Kit ("Pierce"). The DNA-protein complexes were separated from the free DNA probe through 5% nondenaturing polyacrylamide gel electrophoresis (PAGE) in ×0.5 TBE buffer. Then the gel was transferred to a nylon membrane. For competition experiments, 100 fold unlabeled DNA was co-incubated with labeled probes and nuclear extracts.

Transmission electron microscopy. For transmission electron microscopy (TEM), cells were fixed initially in 0.1 M sodium phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde. Next, cells were fixed in 0.1 M sodium phosphate buffer containing 1% OsO_4 (pH 7.2) for 2 h at 4°C, and dehydrated in a graded series of ethanol. Cells were then embedded into Ultracut ("Leica") and sliced into 60 nm sections. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEM-1230 transmission electron microscope ("JEOL").

Flow cytometry. Transfected cells were trypsinized, washed twice with PBS, and resuspended in 200 μl of binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 2.5 mM $CaCl_2$). Fluorescein isothiocyanate (FITC)-conjugated Annexin V was added to a final concentration of 0.5 μg/ml. After incubating for 20 min at room temperature in the dark, propidium iodide (PI) was added at 1 μg/ml, and the samples were immediately analyzed on a FACSCalibur flow cytometer ("Becton Dickinson").

RESULTS

Cloning and bioinformatics analysis of human TSR2

The full-length human *TSR2* cDNA clone (NM_058163) was 1353 base pairs with a 3'-poly(A) tail, and potentially encodes a 191 amino acid protein with a predicted molecular mass of 20.9 kDa and an isoelectric point of 4.04. The full length cDNA and predicted amino acid sequence of TSR2 are shown in Fig. 1*a*. Human TSR2 is located on chromosome Xp11.22, and has five exons and four introns. A database search revealed that TSR2 is conserved in human, *Pan troglodytes*, *Canis familiaris*, *Bos taurus* and *Mus musculus* (Fig. 1*b*). However, no functional studies on human TSR2 have been done.

Expression profile of human TSR2

Northern blot analysis was used to confirm the presence of *TSR2* mRNA in human tissues. As shown in Fig. 2*a*, a band of approximately 1.5 kb was detected in brain, thyroid, larynx, lung and heart samples, which is consistent with bioinformatics analysis. RT-PCR analysis revealed the differences in *TSR2* expression levels between various cell types. *TSR2* mRNA was observed in a variety of cell lines, including 293, Jurkat, Raji and K562 cells. But the expression was weak in HeLa and HEp-2 cells (Fig. 2*b*). In order to further ascertain human *TSR2* expression lev-

a

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                                                                    ta
atg gcg ggc gct gca gaa gat gcg cga gct ctt ttc cgg gct ggg gtc tgc gcg gcc
M   A   G   A   A   E   D   A   R   A   L   F   R   A   G   V   C   A   A
ctg gag gcc tgg ccg gcc ttg cag atc gct gtg gag aat ggc ttc ggg ggt gtg cac
L   E   A   W   P   A   L   Q   I   A   V   E   N   G   F   G   G   V   H
agc cag gag aag gcc aag tgg ctg ggg ggt gca gtg gag gat tac ttc atg cgc aat
S   Q   E   K   A   K   W   L   G   G   A   V   E   D   Y   F   M   R   N
gct gac ttg gag cta gat gag gtg gaa gac ttc ctt gga gag ctg ttg acc aac gag
A   D   L   E   L   D   E   V   E   D   F   L   G   E   L   L   T   N   E
ttt gat aca gtt gtg gaa gac ggg agt ctg ccc cag gtg agc cag caa ctg cag acc
F   D   T   V   V   E   D   G   S   L   P   Q   V   S   Q   Q   L   Q   T
atg ttc cac cac ttc cag agg ggt gat ggg gct gct ctg agg gag atg gcc tcc tgc
M   F   H   H   F   Q   R   G   D   G   A   A   L   R   E   M   A   S   C
atc act cag aga aaa tgc aag gtc aca gcc act gca ctt aag aca gct aga gag act
I   T   Q   R   K   C   K   V   T   A   T   A   L   K   T   A   R   E   T
gat gag gat gaa gat gat gtg gac agt gtg gaa gag atg gag gtc aca gct acg aat
D   E   D   E   D   D   V   D   S   V   E   E   M   E   V   T   A   T   N
gat ggg gct gct aca gat ggg gtc tgc ccc cag cct gaa ccc tct gat cca gac gct
D   G   A   A   T   D   G   V   C   P   Q   P   E   P   S   D   P   D   A
cag act att aag gaa gag gat ata gtg gaa gat ggc tgg acc att gtc cgg aga aaa
Q   T   I   K   E   E   D   I   V   E   D   G   W   T   I   V   R   R   K
aaa tga gtg ggg atg att gga aat ggc ttt ggg ccc tta ttt gct gtt cta aga gtt
K
gtc tgt agg ggt ttt ttt ttt gag gat tgc aga cct gtg gac tgg tta ccc cat ctc
cac cct ctc ccc tgt tcc tct gtc tgg ctc cct cca ggg caa gga aaa cct agg gtc
ctt ggt ctt ggg ggt ggg ggg acc ttt tcc agc ata ttc ccc tgg gcc ttt agt taa
cat ccg agt gac gaa gcc ggg ttc ccc agt aca gtg tct ttt ggg tgc ggt cat tgt
gag gaa ggg gat gag tga gtg tgg gtg tgg ctg gag gag gag cta gat gcc ggt gga
cag tgg gca ggt gga ttt ggg gag gag agg atc agc agc agc agc agt act cag tga
tag ggg gct ggc tgg gtc agg aat ggt gag atg aac atg caa gtg gaa gag aga ata
gtt gaa aat gtg aat gaa agt agc tct gga gcc tct ccc tta ccc cac ccc aca cac
acg caa cag ctc tgt acc cct gcc cag aac ttg gaa cct ccc agg aat gct gcc ttc
ctt cag ccc agc ctc cag tcc tgg gtt ccc tca gga att cga aga cct tgg cta ggt
ggt gag ctt gga aga gct ggg ctt tgt ttt ctg tct cct cta acc tct ttc ctt act
cct tat ccc atc cca ttt gag ttc agg ggt gag gct taa gat ctt aat aaa taa tac
ctt act gct taa aaa aaa aaa aaa aaa aaa aaa aaa aaa a
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b

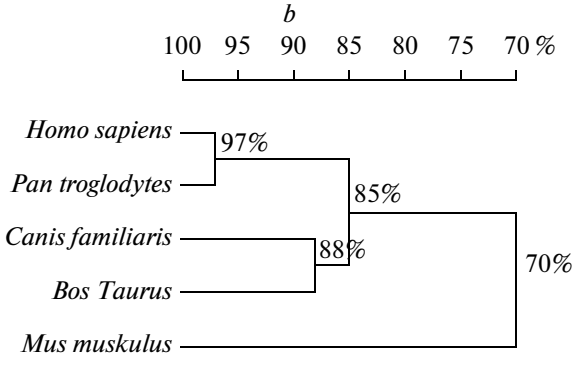

Fig. 1. TSR2 identification and sequence analysis. *a* – Nucleotide sequence and predicted amino acid sequences of the human *TSR2*. Primers used to amplify the ORF are underlined. *b* – Phylogenetic analysis of *TSR2*.

els in those cell lines, we performed real-time PCR; the results confirmed that expression levels in HEp-2 and HeLa were lower than in the other cell lines (Fig. 2*c*). To determine the subcellular localization of TSR2, HEp-2 cells were transiently transfected with TSR2-GFP or an empty GFP vector. Twenty hours after transfection, the transfected cells were observed by light and fluorescence microscopy. Both GFP (Fig. 3*a*) and TSR2-GFP (Fig. 3*b*) cells exhibited diffuse staining throughout the whole cell distribution.

TSR2 inhibits NF-κB transcriptional activity in HEp-2 cells

To investigate the role of TSR2 in cell signal transduction, we examined whether TSR2 is involved in tran-

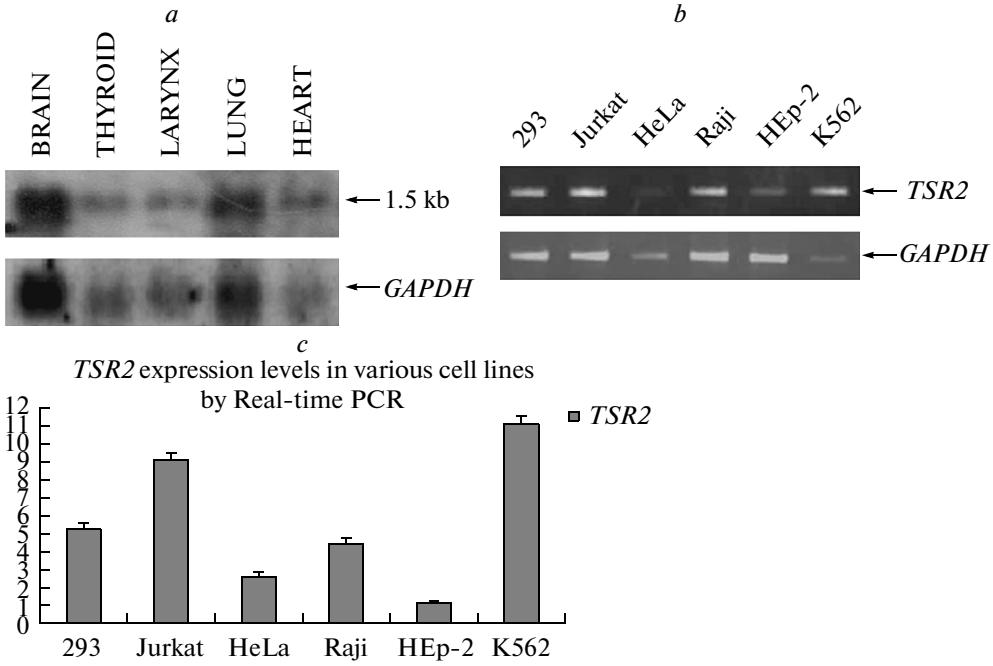

Fig. 2. Expression profile of the human *TSR2*. *a* – Northern blot analysis of *TSR2* expression in human tissues. RT-PCR (*b*) and real-time PCR (*c*) analyses show that *TSR2* is expressed in a variety of cell lines, including 293, Jurkat, Raji and K562 cells.

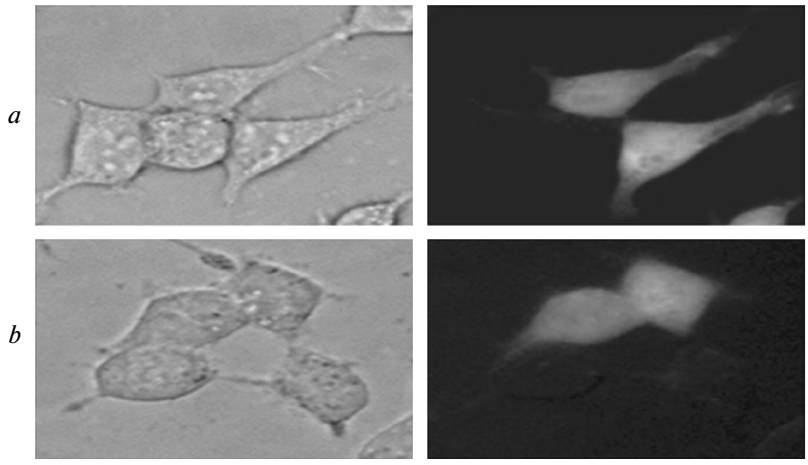

Fig. 3. Subcellular distribution of human TSR2. HEp-2 cells were transfected with GFP (*a*) or TSR2-GFP (*b*) expression vectors and visualized as described in the Experimental section.

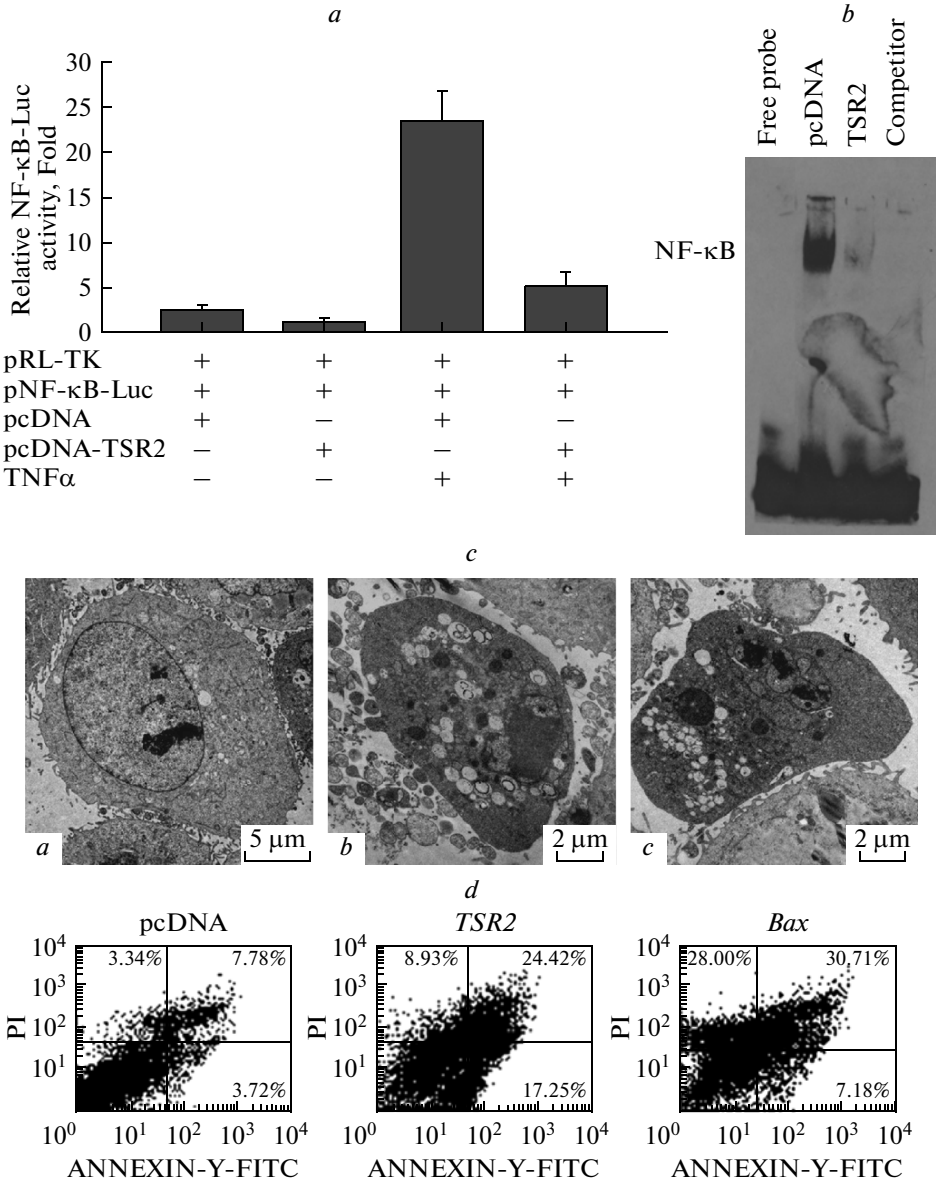

Fig. 4. TSR2 inhibits NF-κB transcriptional activity and induces apoptosis in HEp-2 cells. *a* – TSR2 is a transcriptional suppressor of the NF-κB pathway. HEp-2 cells were stimulated by TNF-α 40 h after transient transfection, and then luciferase activity assays were performed. The relative luciferase activity was shown as fold induction normalized on the signal of the non-stimulated empty vector (pcDNA). *b* – EMSA analysis of NF-κB activation. Compared with the empty vector (pcDNA), TSR2 diminished NF-κB complex binding to the NF-κB probes in HEp-2 cells. *c* – Electron microscopy of TSR2-induced apoptosis: empty vector (a); TSR2 (b) or Bax-transfected HEp-2 cells (c). *d* – *TSR2* overexpression induces apoptosis. Transfected HEp-2 cells were harvested and stained with Annexin V-FITC and PI. The percentage of Annexin V and/or PI positive cells is shown.

scriptional regulation of NF-κB, AP-1 or NFAT. We overexpressed *TSR2* in HEp-2 cells, together with the reporter vectors that allowed to detect the activation or inhibition through the luciferase activity. *TSR2* overexpression significantly suppressed NF-κB reporter gene activity in the absence or presence of TNF-α. As shown in Fig. 4*a*, TNF-α treatment increased NF-κB luciferase activity significantly. The NF-κB activity induced by TSR2 in cells treated with TNF-α was inhibited markedly in comparison to the empty pcDNA vector (approximately 22% of the empty vector activity). The transcriptional activity of AP-1 and NFAT were not influenced by *TSR2* overexpression (data not shown).

EMSA analysis was used to assess the effect of TSR2 on binding of proteins from the nuclear extract to NF-κB specific probes. As shown in Fig. 4*b*, TNF-α markedly induced a NF-κB complex specific retardation band in the empty vector (pcDNA) or *TSR2* transfected cells. The free probe or excess of unlabeled competitor probe totally eliminated the NF-κB complex specific band,

confirming the specificity of interaction. Compared with the empty vector (pcDNA), TSR2 diminished NF-κB complex binding to NF-κB probes in HEp-2 cells. These results, combined with the luciferase reporter assay, suggest that *TSR2* overexpression may inhibit NF-κB translocation into the nucleus or NF-κB binding to DNA.

TSR2-induced cell death involves hallmarks of apoptosis

We observed the morphological changes characteristic for apoptosis in TSR2-transfected HEp-2 cells. As shown in Fig. 4*c*, we used TEM to reveal typical apoptotic features, including condensed chromatin at the nuclear periphery (part b, Fig. 4*c*), similar to Bax-transfected cells (part c, Fig. 4*c*). Empty vector pcDNA-transfected cells had a normal phenotype (part a, Fig. 4*c*). A key biochemical hallmark of apoptotic cell death is the translocation of PS from the cytoplasmic surface of the cell membrane to the external cell surface [16]. Exposure of phosphatidylserine (PS) at the surface of apoptotic cells can be easily identified by flow cytometry using fluorescence-labeled Annexin V, which specifically binds PS. Using this assay, we detected PS on the surface of HEp-2 cells by FITC/Annexin V staining 40 h after transfection with TSR2. Plasma membrane integrity was assessed simultaneously by PI exclusion using two-color flow cytometry. As shown in Fig. 4*d*, in HEp-2 cells transfected with *TSR2*, the portion of Annexin V and/or PI positive cells was increased in comparison with pcDNA-transfected cells. These results demonstrated that *TSR2* overexpression induced apoptosis.

DISCUSSION

In the present study, we cloned the full length ORF of the human gene *TSR2*. TSR2 is conserved in humans, chimpanzee, dogs, cattle and mice, indicating that it has important functions in vertebrate animals. Northern blot results revealed that TSR2 is broadly and moderately expressed in several tissues, including the larynx. Transfecting *TSR2* into HEp-2 cells significantly inhibited the transcriptional activation of NF-κB in the absence or presence of TNF-α. Moreover, TSR2 overexpression induced apoptosis, characterized by nuclear condensation and PS externalization. These findings for the first time showed that TSR2 may inhibit the NF-κB signaling pathway to mediate apoptosis.

Recent lines of evidence have indicated that the transcription factor NF-κB is constitutively active in many tumors, and that this abnormal activity contributes to carcinogenesis [17–20]. Therefore, identifying the mechanisms for switching off aberrant NF-κB activity could have a major therapeutic benefit [21–23]. In the current study, we found that overexpression of TSR2 inhibited NF-κB transcriptional activity, as determined by luciferase reporter assay and EMSA. But TSR2 did not influence other signaling pathways, such as NFAT and AP-1 (data not shown). Studies have shown that NF-κB suppression promotes apoptosis [24, 25]. We found hallmarks of apoptosis in *TSR2*-transfected HEp-2 cells, including chromatin condensation and PS externalization. Our data indicates that TSR2 may have a role in apoptosis through the NF-κB pathway. To date, considerable progress has been made in elucidating the relationship of the NF-κB pathway and apoptosis. Based on our previous functional studies on TSR2, we speculate that TSR2 may inhibit phosphorylation and degradation of IκBα to influence the nuclear translocation of the NF-κB complex (p65/p50), which reduces the expression of NF-κB regulated genes, such as the anti-apoptotic genes (IAPs, XIAP, Bcl-2 or Bcl-xL), thus inducing apoptosis.

Further investigations are needed in order to identify the precise relationship between NF-κB inhibition and TSR2-induced apoptosis, as well as the molecular mechanism by which TSR2 induces apoptosis. In conclusion, our results for the first time have shown that TSR2 is involved in the NF-κB signaling pathway and apoptosis. This may aid in the effort to develop a new strategy of therapies for cancers, and especially the laryngeal cancer.

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