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Identification of β1,4GalT II as a Target Gene of p53-mediated HeLa Cell Apoptosis

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β1,4-galactosyltransferase II (β1,4GalT II) is one of the enzymes transferring galactose to the terminal *N*-acetylglucosamine of complex-type *N*-glycans. Previously, we have reported that β1,4GalT II overexpression increased cisplatin-induced HeLa cell apoptosis. However, the mechanisms of its expression regulation have been rarely investigated. Here, we cloned the 1.8-kb 5'-flanking region of the β1,4GalT II gene and analysed its promoter activity. The transcriptional activity and mRNA expression level of β1,4GalT II were dramatically induced by p53 transcription factor in HeLa cells. In response to DNA damage agent adriamycin, the mRNA expression and promoter activity of β1,4GalT II were significantly up-regulated and the binding of p53 to β1,4GalT II promoter was obviously increased. Furthermore, decreasing the expression of β1,4GalT II using RNA interference inhibited p53-mediated HeLa cell apoptosis induced by adriamycin. Collectively, these results suggested that β1,4GalT II might serve as a target gene of p53 transcription factor during adriamycin-induced HeLa cell apoptosis, which elucidated a new mechanism of p53-mediated cell apoptosis.

Key words: adriamycin, apoptosis, β1,4-galactosyltransferase II, p53, transcription regulation.

Abbreviations: ChIP, chromatin immunoprecipitation; β1,4GalT II, β1,4-galactosyltransferase II; RCA-I, *Ricinus communis* agglutinin-I; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; SDS-PAGE, SDS-poly-acrylamide gel.

Apoptosis, which is a common cellular response to stress caused by environmental challenges, plays critical roles in cancer chemotherapy (*1*). Cells die from cancer chemotherapy due to apoptosis largely controlled by wild-type p53 (*2*). The requirement for wild-type p53 for apoptosis after genotoxic damage caused by anti-cancer agents has been well demonstrated (*3*, *4*). However, the mechanisms of p53-mediated cell apoptosis induced by DNA damage agents need further investigations.

N-glycans of cell surface glycoprotein is important for the regulation of cell apoptosis, survival and proliferation (*5*–*7*). β1,4-galactosyltransferase (β1,4GalT) family are the enzymes responsible for the biosynthesis of *N*-acetyllactosamine on *N*-glycans by transferring UDP-galactose to the terminal *N*-acetylglusamine (*N*-GlcNAc) residues (*8*, *9*). β1,4GalT II, a member of β1,4-galactosyltransferase family, could transfer galactose to the terminal *N*-acetylglucosamine of complex-type *N*-glycans (*10*). Our recent study provided evidence that the mRNA expression level of β1,4GalT II was altered during cell apoptosis induced by DNA damage agents and its overexpression increased cisplatin-induced HeLa cell apoptosis (*11*). However, little is known about the mechanism of transcription regulation of β1,4GalT II.

To address this point, we cloned the 1.8-kb 5′-flanking region of the β1,4GalT II gene. Luciferase assay and RT–RCR analysis indicated that p53 was an important regulator of β1,4GalT II transcription. During adriamycin-induced cell apoptosis, the binding of p53 to β1,4GalT II promoter was obviously increased, resulting in activation of β1,4GalT II transcription. Furthermore, decreasing the expression of β1,4GalT II using RNA interference inhibited p53-increased HeLa cell apoptosis induced by adriamycin. Collectively, p53 might regulate adriamycin-induced cell apoptosis, at least, partly through up-regulation of β1,4GalT II transcription, indicating a new mechanism of p53-mediacted cell apoptosis.

MATERIALS AND METHODS

Materials—Restriction enzymes, bovine calf serum, Dulbecco's modified Eagle's medium (DMEM) medium, Trizol reagent and Lipofectamine reagent were purchased from Invitrogen. Hechest33258 and adriamycin were purchased from Sigma Chemical. The anti-GFP and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology.

Cell Culture and Cell Transfection—HeLa cells were cultured inDMEM. Cell transfection was performed with Lipofectamine (Invitrogen), according to the manufacturer's instructions.

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Analysis of Apoptosis by Fluorescence Staining and Flow Cytometry and Western Blot Analysis—Fluorescence staining and flow cytometry were performed as previously described (*12*, *13*). Western blots were performed as previously described (*14*).

Dual Luciferase Assay and Reverse Transcription (RT)–PCR—Dual luciferase assay and RT–PCR were performed as previously described (*14*). Primers used for PCR were as follows: β1,4GalT II sense 5′-cggtcatc atccccttaga-3′ and anti-sense 5′-attggtgaagagtggttgcc-3′, β1,4GalT I sense 5′-atgaggcttcgggagccgctcctg-3′ and anti-sense 5′-ctagctcggtgtcccgatgtc-3′, β1,4GalT III sense 5′-gtcctgtgtcggtgtccttt-3′ and anti-sense 5′- gtacggaccag gaggtcaaa-3′, β1,4GalT IV sense 5′-gctgttgactttgtgcctga-3′ and anti-sense 5′-ggagctcaaccctgagtctg-3′, β1,4GalT V sense 5′-tgagaacaatcggtgctcag-3′ and anti-sense 5′- ctcac tccgccaaagaactc-3′, β1,4GalT VI sense 5′-ccctctcttc gtcctgtctg-3′ and anti-sense 5′- aattgttccactgtcagccc-3′, β1,4GalT VII sense 5′-acgatgttcccctcgcggagg-3′ and anti-sense 5′-ccatccagctcagctgaatgt 3′, β-actin sense 5′-atgggt cagaaggattcctat-3′ and anti-sense 5′- gcgctcggtgaggatct tcat-3′.

RNA Ligase-Mediated Rapid Amplification of the 5′ cDNA End—5′-RACE analysis was performed to map the transcription start site using a FirstChoice RLM-RACE kit (Ambion) according to the manufacturer's instructions. The 5′ RACE Adapter (5′-GCUGAUGGCGAU GAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3′) was ligated to the 5′-ends of the decapped mRNAs from HeLa cells. Subsequently, the cDNAs were synthesized with M-MLV Reverse Transcriptase. The 5′-RACE primer (5′-GCTGATGGCGATGAATGAACACTG-3′) and β1,4GalT II gene specific primer (GSP) (5′-TCCACAT CGCTGAAGATGAAGC-3′) were used in the PCR under the following conditions: 94°C, 30 s; 55°C, 50 s; and 72°C, 150 s. The products were subjected to electrophoresis in a 1.5% agarose gel and visualized under a UV lamp. As a final product, a DNA fragment comprising ~500 bp was obtained, cloned into pMD19-T vector and sequenced.

Plasmids—Expression constructs for pRL-CMV, pSilencer-2.0, pGL3-SV40, E1AF, Ets1, Ets2, Sp1, Elk1, Net and pGL3Basic have been described previously (*12*, *14–16*). Expression constructs for p53 was generous gift from Dr. Bert Vogelstein (The John Hopsking Oncology Center). A 1,800-bp fragment (containing nucleotides from −1,800 to +6) of β1,4GalT II promoter was prepared by PCR amplification of human genomic DNA using primers (*β1,4GalT II*, forward 5′-TATCTCGAGCTCCAGTGATGG TCCTGTCCTTCT-3′, and reverse 5′-TATAAGCTTCTGA GCAGGGTGGGAGGGAAA-3′). Following digestion with restriction enzymes, the β1,4GalT II fragment was directionally cloned into Xho I/HIND III-digested pGL3-basic vector to generate a full-length β1,4GalT II promoter construct pGL3(−1,800/+6). Reporter constructs pGL3(−1,600/+6), pGL3(−1,400/+6), pGL3(−1,200/+6), pGL3(−1,000/+6), pGL3(−500/+6) and pGL3(−400/+6) were prepared in the similar manner. Construction of β1,4GalT *II* RNAi was performed using siRNA construction kit (KCsiRNA) according to the manufacture's suggestions (*14*). The sequences of β1,4GalT II mRNA target oligonucleotides were as follows: RNAi 1, GGCTCAGTTTCTGAGAATC; RNAi 2, CACGAAGCTG ACCATGAAG; RNAi 3, TATCACAGTGGACATTGGG.

Chromatin Immunoprecipitation Assays—The association of p53 with β1,4GalT II and p21 chromatin DNA in HeLa cells treated with or without adriamycin was confirmed using a CHIP assay kit (Upstate Biotechnology) with anti-p53 Ab as described by the manufacturer. Normal anti-mouse IgG was used as a negative control. The β1,4GalT II promoter region (−400/−1) was amplified by conventional PCR. The p21 promoter region containing a p53-binding site was amplified as previously reported (*3*).

Lectin Blotting Analysis—Total extracts from cells were analysed by lectin blotting assay with RCA-I as our previous report (*12*). The expression of GAPDH served as a loading control.

RESULTS

Mapping of the Transcription Start Site—To determine the position of the transcription start site of the human β1,4GalT II gene, 5′-RACE analysis was performed using total RNA from HeLa cells and two sets of oligonucleotide primers, 5′-Race Primer and GSP (β1,4GalT II gene specific primer) (Fig. 1A). As shown in Fig. 1B, the PCR produced a 500-bp product. The product was extracted from the agarose gel and cloned into pMD19-T vector for sequencing. The result showed that the RNA Oligo was linked to an cytosine residue at nucleotide position 7-bp upstream from the initiation codon (Fig. 1C), indicating that the transcription of the β1,4GalT II gene started at this position.

Sequence and Functional Analysis of the β1,4GalT II Gene Promoter—The 5′-flanking region of the β1,4GalT II gene was cloned to analyse its promoter function. Computer analysis of the human β1,4GalT II promoter revealed a high GC-rich content in its promoter region. The TRANSFAC search program predicted a number of potential transcription factor-binding sites near or upstream of the putative transcription initiation site, including Ets1, Ets2, Elf, E1AF, p53 and Sp1 (Fig. 2).

To characterize the *cis*-elements of the β1,4GalT II gene promoter, seven reporter plasmids containing the promoter in variable lengths (Fig. 3A, left panel), were constructed and transfected into HeLa cells and the promoter activities were determined. The results showed that pGL3(−1,800/+6) showed significant luciferase activity (Fig. 3A, right panel), indicating that this fragment contained a functional eukaryotic promoter activity in HeLa cells. Similar results were obtained in HepG2 and MDA231 cell lines (data not shown). In addition, deletion of the region between −1,800 and −1,600, 1,200 and −1,000 or −400 and +6 within β1,4GalT II promoter decreased the promoter activity (Fig. 3A, right panel), indicated that these regions contained positive regulatory element.

Identification of Transcription Factors Regulate the β1,4GalT II Gene Promoter—To determine the transcription factors which contributed in the transcription regulation of β1,4GalT II, pGL3(−1,800/+6) was transiently co-transfected into HeLa cells with a variety of

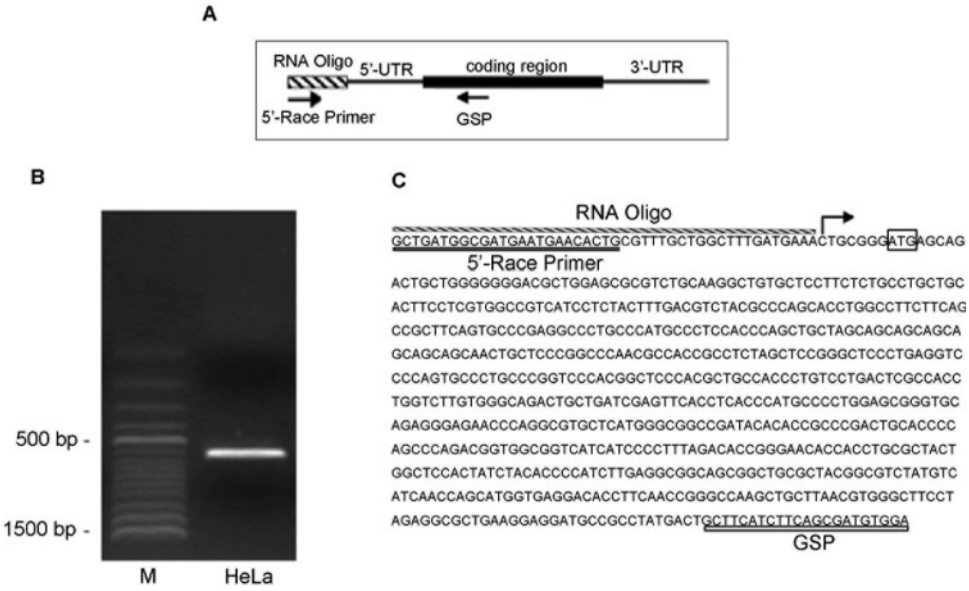

Fig. 1. **5′RACE analysis of the transcription start site of the human β1,4GalT II gene.** (A) Schematic representation of the primers used in RLM–RACE analysis. 'UTR' indicated the untranslated region. The 5′-Race Primer and GSP (β1,4GalT II gene specific primer) were indicated. (B) Ethidium bromide staining of the PCR products on 1.5% agarose gel. The 5′RACE Race primer and GSP (β1,4GalT II gene specific primer) were used in the PCR as described in MATERIALS AND METHODS section. M indicated the 100-bp DNA ladder used as a molecular size marker. (C) Nucleotide sequence of the PCR product. The arrow indicated the transcription start site, and the translation initiation codon was indicated by the box. The complementary sequence for 5′-Race Primer and GSP (β1,4GalT II gene specific primer) used in the PCR was underlined.

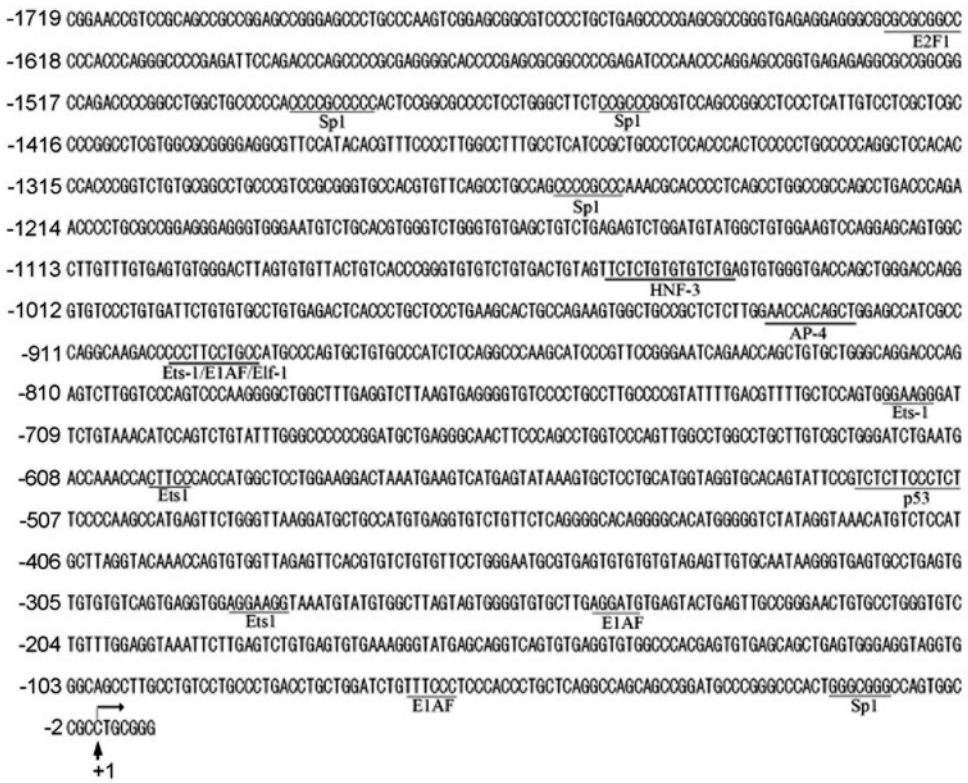

Fig. 2. **Nucleotide sequence of the 5′-flanking region of the β1,4GalT II gene.** Numbers at the left referred to the transcription start site, which was indicated with an arrow and taken as +1, as determined by RLM–RACE analysis in the present study. Potential transcription factor binding sites identified by searching TRANSFAC transcription factor database were underlined and labelled.

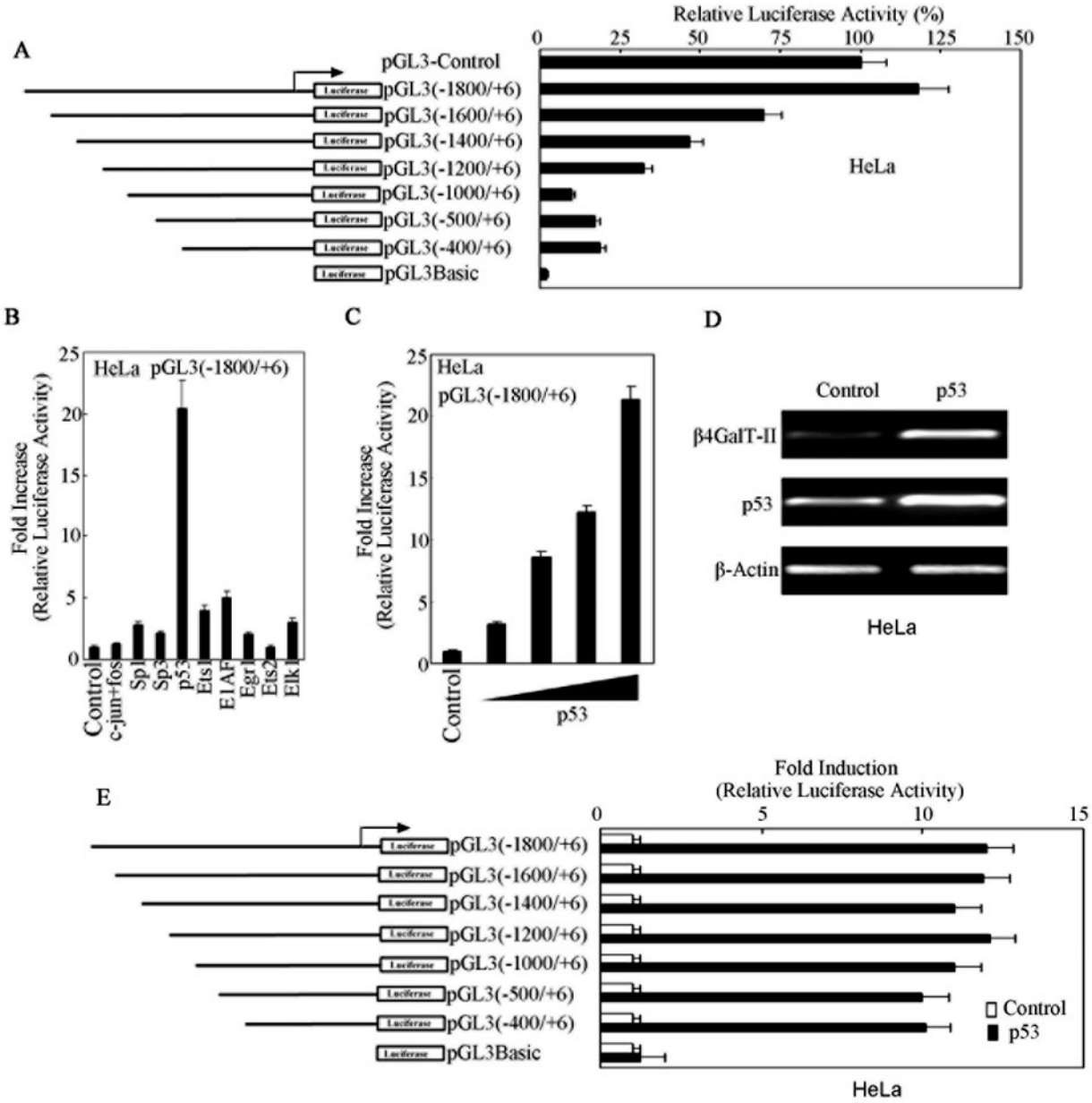

Fig. 3. **The effect of p53 on β1,4GalT II transcription.** (A) A series of the 5′-deleted β1,4GalT II promoter fragments were fused to the luciferase reporter gene. The arrow indicated the transcription start site (left panel). HeLa cells were transiently transfected with the indicated promoter construct and the luciferase activity was determined 48 h after transfection. Transfection efficiency was adjusted by co-transfection with pRL-CMV and parallel transfections with pGL3-SV40 and pGL3-Basic, used as positive and negative control, respectively. The promoter activity of pGL3-SV40 was taken as 100%. Three experiments were conducted and the data were shown as the mean values with standard errors. (B) PGL3(−1,800/+6) was transiently transfected into HeLa cells with the indicated expression vector and the luciferase activity was determined 48 h after transfection. Results shown were the means ± SD of at least three independent experiments, expressed relative to activity in control cells transfected with empty expression vectors. (C) PGL3(−1,800/+6) was transiently transfected into HeLa cells with an increasing amounts of p53 expression vectors and the luciferase activity was determined 48 h after transfection as described above. (D) RT–PCR assay was performed to investigate the mRNA expression of β1,4GalT II gene from HeLa cells transiently transfected with control or p53 expression vector using primers specific to the β1,4GalT II and p53. Level of β-actin mRNA expression served as a loading control. (E) HeLa cells were transiently co-transfected with pGL3(−1,800/+6) construct or the truncated β1,4GalT II promoter constructs shown above, and with or without p53 expression vector. The luciferase activity was determined 48 h after transfection as described above.

transcription factors or the empty control. The highest activation of β1,4GalT II promoter was obtained by p53 (Fig. 3B). To further determine the effect of p53 on the β1,4GalT II transcription, pGL3(−1,800/+6) was transiently co-transfected into HeLa cells with increasing amounts of p53 expression plasmids. Forced expression of p53 increased the activity of β1,4GalT II promoter in a dose-dependent manner in HeLa cells (Fig. 3C). Similar

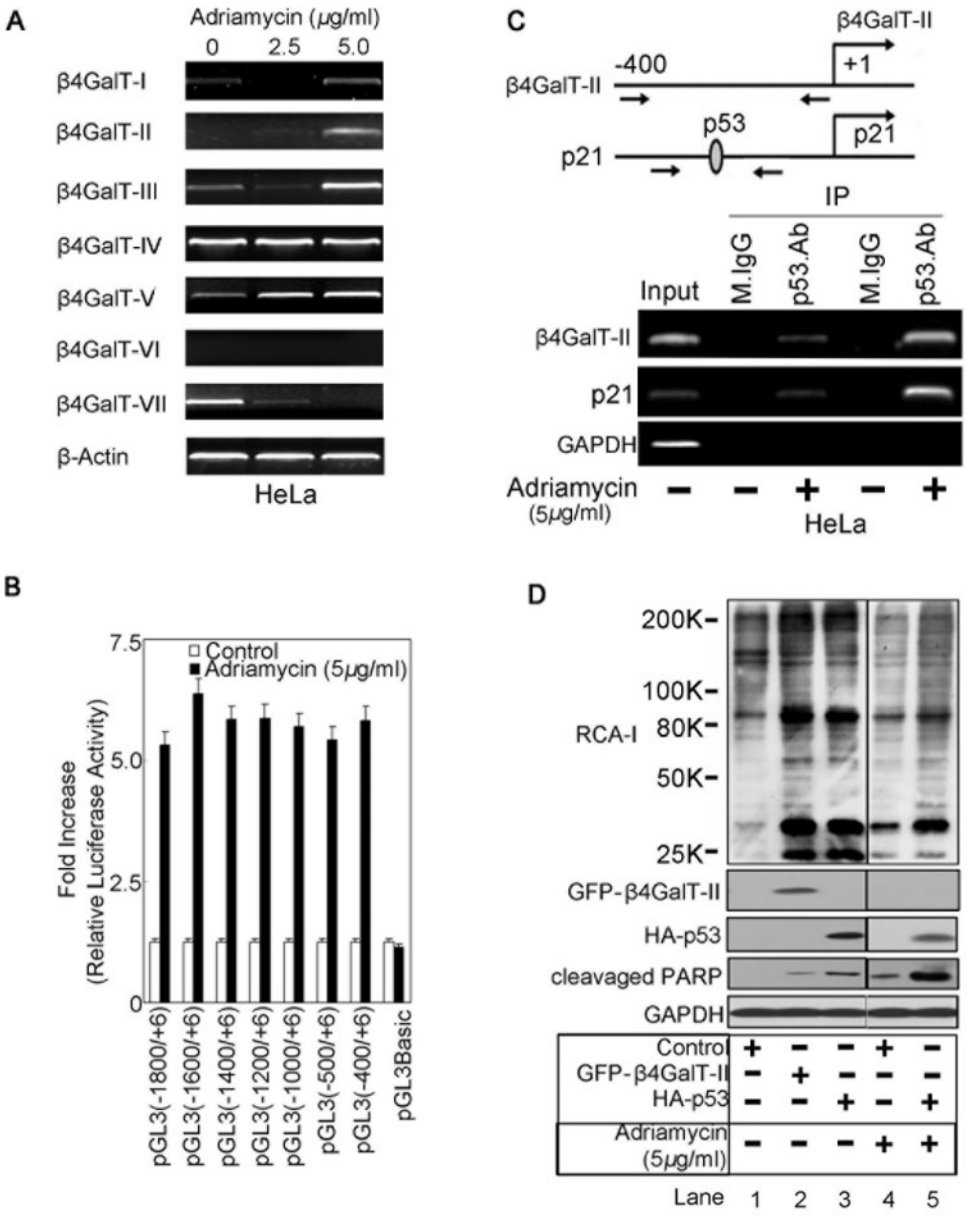

Fig. 4. **The effect of adriamycin on β1,4GalT II mRNA expression and transcription.** (A) The mRNA expression of β1,4GalT family member in HeLa cells untreated or treated with adriamycin for 24 h in the indicated dose was analysed by RT–PCR. The mRNA expression of β-Actin served as a loading control. (B) HeLa cells transiently transfected with pGL3(−1,800/+6) construct or the truncated β1,4GalT II promoter constructs were treated with adriamycin (5 μg/ml) for 24 h and the luciferase activity was determined 48 h after transfection as described above. Results shown were the means ± SD of at least three independent experiments, expressed relative to activity in control cells. (C) CHIP assay was performed using HeLa cells treated with or without adriamycin (5 μg/ml) for 24 h and control mouse IgG (M. IgG) or an antibody against p53. PCR primers for the β1,4GalT II promoter or the p21 promoter was used to detect promoter fragments in immunoprecipitates. (D) Lectin blot analysis of protein from HeLa cells transiently transfected with control, GFP-tagged β1,4GalT II or HA-tagged p53 and treated with or without adriamycin (5 μg/ml) for 24 h. Proteins were separated by SDS–PAGE and analysed by RCA lectin as described in MATERIALS AND METHODS section. The expression of GFP-β1,4GalT II or HA-p53 was indicated and the expression of GAPDH served as a loading control.

result was obtained in H1299 cells (data not shown). Consistent with this, compared to control, the mRNA expression of β1,4GalT II was dramatically increased in p53-transfected HeLa cells (Fig. 3D). To identify the *cis*-element responsible for the effect of p53 on β1,4GalT II promoter, HeLa cells were transiently co-transfected with pGL3(−1,800/+6) construct or the truncated β1,4GalT II promoter constructs and with or without p53 expression vector The luciferase assay showed that a deletion from −400 to +6 resulted in a loss of p53 activation (Fig. 3E), suggesting that the potential p53-binding site between nucleotide −519 and −508 was not response for the activation of β1,4GalT II transcription by p53.

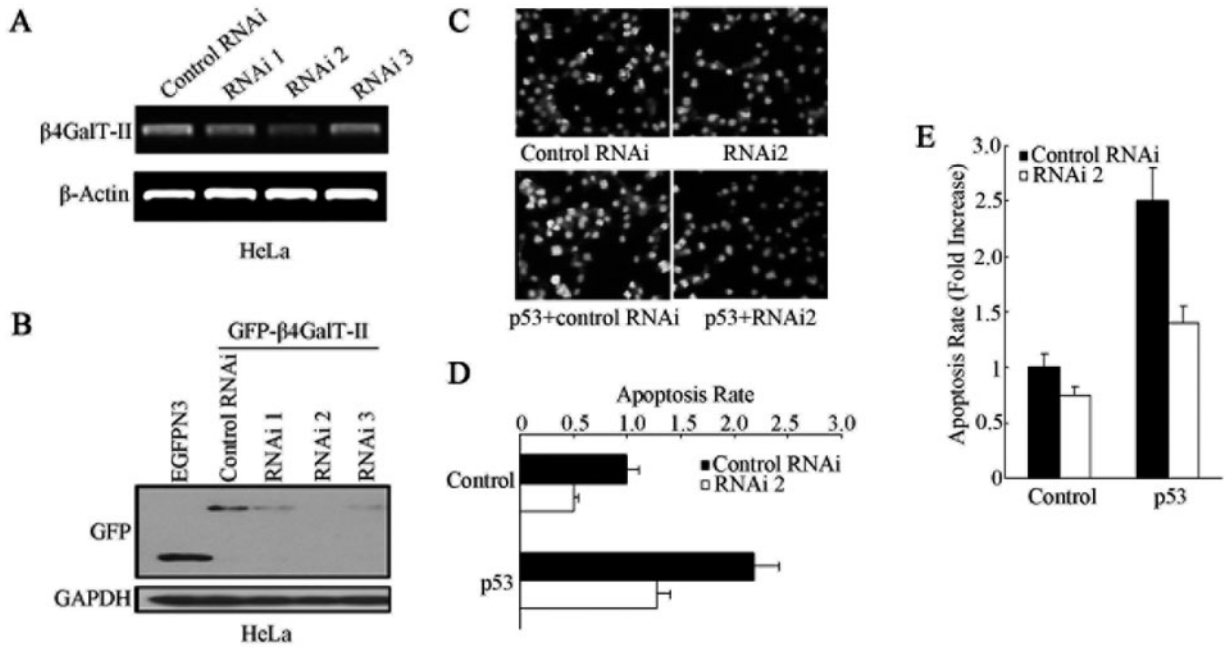

Fig. 5. **The role of β1,4GalT II in p53-mediated apoptosis induced by adriamycin.** (A) RT–PCR analysis of β1,4GalT II mRNA expression level in HeLa cells transiently transfected with control RNAi, RNAi 1, RNAi 2 or RNAi 3. Level of β-actin mRNA expression was assessed as a loading control. (B) Western blot analysis of the expression of GFP or GFP-β1,4GalT II from HeLa cells transiently co-transfected with GFP or GFP-β1,4GalT II with control RNAi, RNAi 1, RNAi 2 or RNAi 3. GAPDH expression served as a loading control. (C) Hoechst 33258 staining of nuclei from HeLa cells transiently transfected with control, and/or p53, and/or control RNAi, and/or β1,4GalT II RNAi plasmids and treated with or without adriamycin. (D) At least 300 cells were counted from three different microscope fields and the percentage of apoptosis was standardized with that of HeLa cells untreated with adriamycin (5 μg/ml) for 24 h. Each value was the mean ± SD of at least three independent experiments. (E) HeLa cells transiently transfected with control, and/or p53, and/or control RNAi, and/or β1,4GalT II RNAi plasmids were harvested after the treatment of adriamycin (5 μg/ml), fixed in ethanol and stained with propidium iodide. The apoptotic rates were counted by flow cytometry analysis. Each value was the mean ± SD of at least three independent experiments.

The Effect of Adriamycin on β1,4GalT II mRNA Expression and Promoter Activity—P53 plays a critical role in triggering apoptosis induced by DNA damage agent adriamycin (*2*, *4*). To investigate the contribution of β4GalT II in adriamycin-induced cell apoptosis, we first investigated the effect of adriamycin on β1,4GalT II mRNA expression. As depicted in Fig. 4A, adriamycin significantly increased the mRNA expression of β4GalT II and β4GalT V and decreased the mRNA expression of β4GalT-VII in a dose-dependent manner. To confirm that the up-regulation of β4GalT II expression by adriamycin was at the transcriptional level, luciferase reporter constructs containing progressive deletion of the 1,800-bp genomic DNA fragment were transiently transfected into HeLa cells. As expected, adriamycin enhanced β4GalT II promoter activity and a deletion from −400 to +6 resulted in a loss of adriamycin activation (Fig. 4B). To investigate the contribution of p53 in adriamycin-induced β4GalT II transcription, CHIP was performed to investigate the binding of p53 to β4GalT II promoter in response to adriamycin. Compared to adriamycin-untreated cells, the bindings of p53 to β4GalT II promoter and p21 promoter were obviously increased in response to adriamycin (Fig. 4C). Due to the correlation between β4GalT II and galactose synthesis, we performed lectin blot analysis to investigate whether the galactosylated *N*-glycans synthesized by β4GalT II were involved in this p53-mediated apoptosis of HeLa cells by the treatment with adriamycin. Compared to the mock-transfected cells, a significant increase of the binding of total glycoprotein with Ricinus communis agglutinin-I (RCA-I) was observed for 25–50-kDa and 80–100-kDa protein bands in HeLa cells transiently transfected with β4GalT II or p53 expression plasmids (Fig. 4D, lane 1–3). Similarly, a remarkable increase in the lectin binding was observed for 25–50-kDa and 80–100-kDa protein bands in the cells transfected with p53 expression vector in adriamycin-treated HeLa cells, compared to control (Fig. 4D, lanes 4 and 5).

Reducing the Expression of β1,4GalT II Inhibited p53-Mediated Cell Apoptosis—To address the role of β1,4GalT II in p53-mediated cell apoptosis, we designed and synthesized three different duplex siRNAs complementary to human β1,4GalT II mRNA. Compared to the control RNAi, RNAi 2 specifically suppressed endogenous β1,4GalT II mRNA expression in HeLa cells (Fig. 5A). Consistent with this, compared to the control RNAi, RNAi 2 specifically suppressed exogenous GFP-β1,4GalT II expression (Fig. 5B).

To investigate the contribution of *β4GalT II* in p53-mediated cell apoptosis, HeLa cells transiently transfected with control RNAi, and/or RNAi 2, and/or p53 were treated with adriamycin for 24 h, following Hoechst staining assay. As shown in Fig. 5C and D, compared to

the controls, decreasing β*4GalT II* inhibited p53-increased cell apoptosis induced by adriamycin. The similar results were obtained by FACS assay (Fig. 5E). Collectively, these results suggested that β1,4GalT II might be a target gene of p53-mediated cell apoptosis.

DISCUSSION

β1,4-galactosyltransferase II (β*4GalT II*) is one of the enzymes transferring galactose to the terminal *N*-acetylglucosamine of complex-type *N*-glycans (*9*, *10*). Its mRNA expression was significantly altered during oncogenesis (*17*). Recent data indicated that the expression of β*4GalT II* altered during cell apoptosis induced by DNA damage (*11*, *18*), and β*4GalT II* overexpression increased HeLa cell apoptosis induced by cisplatin (*11*). These data indicated that β*4GalT II* contributed to cell apoptosis induced by DNA damage. However, the mechanisms of its expression regulation have been rarely investigated.

Here, we investigated the mechanisms of transcription regulation of β1,4GalT II. The promoter of β1,4GalT II lacked a typical TATA box, as seen with many GC-rich elements, which was similar to that of β1,4GalT I or β1,4GalT V gene (*19*, *20*). Sp1 transcription factor binds to GC box motifs in promoter of numbers genes involved in growth regulation and cancer (*21*). Sp1 overexpression induced the activity of β1,4GalT II promoter and the mRNA expression level of β1,4GalT II (data not shown), which indicated that β1,4GalT II was a target gene of Sp1 transcription factor. In addition, we found that p53 overexpression increased the promoter activity and mRNA expression level of β1,4GalT II. Luciferase assay indicated the region between nucleotide −400 to +6 in β1,4GalT II promoter was critical for activation by p53, suggesting that the potential p53-binding site between nucleotide −519 and −508 was not response for the activation of β1,4GalT II transcription by p53. CHIP analysis indicated that p53 could bind to the region between nucleotide −400 and −1 of β1,4GalT II promoter. As stated above, inspection of this region revealed no potential p53-binding site. These data suggested that p53 might regulate β1,4GalT II transcription *via* interaction with other transcription factor, which could directly bind to β1,4GalT II promoter.

Another important finding was the contribution of β1,4GalT II in p53-mediated cell apoptosis induced by adriamycin. p53 transcription factor is widely known to activate vital damage-containment procedures to restrict aberrant cell growth and trigger cell apoptosis in response to DNA damage (*3*, *4*, *22*, *23*). More and more evidence indicated that p53 plays a critical role in cell apoptosis induced by adriamycin (*24–26*), which is widely used in the treatment of solid tumours (*27*, *28*). The expression of β1,4GalT family genes were analysed in HeLa cells treated with or without adriamycin. It was found that in response to adriamycin, β1,4GalT II and V was significantly increased and β1,4GalT VII was obviously decreased. Consistent with this, the promoter activity of β1,4GalT II was increased by adriamycin. ChIP analysis showed that the binding of p53 to β1,4GalT II promoter was obviously increased in response to adriamycin, accounting for the elevated expression of β1,4GalT II in response to adriamycin. Furthermore, decreasing the expression of β1,4GalT II inhibited p53-mediated apoptosis induced by adriamycin. Taken all data together, activation of β1,4GalT II transcription by p53 might contribute to adriamycin-induced cell apoptosis. In addition, Sato *et al*. (*19*) have shown that β1,4GalT V gene expression was induced by carcinogenesis. These data indicated that the members of β1,4GalT family could be induced by different inductions and might contribute to cell behaviour by substrate specificity.

In summary, we identified the transcription start site of β1,4GalT II gene in HeLa cells and found that β1,4GalT II serves as a target of p53 in adriamycin-induced apoptosis. The mechanism of β1,4GalT II-promoted cell apoptosis by adriamycin should be further investigated.

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