Notch1 Signaling Sensitizes Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis in Human Hepatocellular Carcinoma Cells by Inhibiting Akt/Hdm2-mediated p53 Degradation and Up-regulating p53-dependent DR5 Expression

Notch signaling plays a critical role in regulating cell proliferation, differentiation, and apoptosis. Our previous study showed that overexpression of Notch1 could inhibit human hepatocellular carcinoma (HCC) cell growth by arresting the cell cycle and inducing apoptosis. HCC cells are resistant to apoptotic induction by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), so new therapeutic approaches have been explored to sensitize HCC cells to TRAIL-induced apoptosis. We are wondering whether and how Notch1 signaling can enhance the sensitivity of HCC cells to TRAIL-induced apoptosis. In this study, we found that overexpression of ICN, the constitutive activated form of Notch1, up-regulated p53 protein expression in HCC cells by inhibiting proteasome degradation. p53 up-regulation was further observed in human primary hepatocellular carcinoma cells after activation of Notch signaling. Inhibition of the Akt/Hdm2 pathway by Notch1 signaling was responsible for the suppression of p53 proteasomal degradation, thus contributing to the Notch1 signaling-mediated up-regulation of p53 expression. Accordingly, Notch1 signaling could make HCC cells more sensitive to TRAIL-induced apoptosis, whereas Notch1 signaling lost the synergistic promotion of TRAIL-induced apoptosis in p53-silenced HepG2 HCC cells and p53-defective Hep3B HCC cells. The data suggest that enhancement of TRAIL-induced apoptosis by Notch1 signaling is dependent upon p53 up-regulation. Furthermore, Notch1 signaling could enhance DR5 expression in a p53-dependent manner. Taken together, Notch1 signaling sensitizes TRAIL-induced apoptosis in HCC cells by inhibiting Akt/Hdm2-mediated p53 degradation and up-regulating p53-dependent DR5 expression. Thus, our results suggest that activation of Notch1 signaling may be a promising approach to improve the therapeutic efficacy of TRAIL-resistant HCC.

Notch signaling determines cell fate and affects cell proliferation, differentiation, and apoptosis during cell development (1). As a highly conserved family, Notch coordinates a signaling cascade present in all animal species studied to date (2). Mammals have four Notch receptors that bind five different ligands, among which Notch1 signaling functions in many physiological and pathophysiological processes of numerous cell types, and its dysfunction results in a variety of developmental defects, including embryonic lethality and adult disorders. For example, the Notch1/Jagged1 signaling pathway is activated during liver regeneration and is potentially contributing to signals affecting hepatocyte growth (3, 4). Inducible inactivation of Notch1 has been shown to cause nodular regenerative hyperplasia in mouse liver (5). These studies suggest that Notch1 signaling may be involved in the liver functions and the pathogenesis of liver diseases. Our previous study demonstrated that Notch1 signaling could suppress the growth of human hepatocellular carcinoma (HCC) cells by arresting the cell cycle and inducing apoptosis (6). However, the underlying molecular mechanisms remain to be fully understood.

p53, an important tumor suppressor gene, is involved in cell cycle arrest and cellular apoptosis. Its activity is mostly regulated by complex networks of post-translational modifications, including phosphorylation, ubiquitination, and proteasome degradation. One protein that is essential for determining p53 stability is Mdm2 (mouse double minute protein 2) (7). Mdm2, a nuclear phosphoprotein and an E3 ubiquitin ligase, binds to p53 and ubiquitinitates p53, leading to proteosome degradation of p53 (8). Another important mechanism of p53 stability is related to its phosphorylation status, which is Mdm2-dependent or Mdm2-independent (9). As to the regulation of...
p53 by Notch1, there are controversial reports that Notch1 activation increased p53 expression in neural progenitor cells (10); however, suppression of p53 by Notch signaling was also well established in lymphomagenesis (11). We also reported that Notch1 signaling significantly up-regulated p53 expression in SMMC7721 HCC cells (6); however, the molecular mechanisms remained unclear and needed to be further characterized.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of a superfamily of cell death–inducing ligands, induces apoptosis in a broad range of transformed cells and tumor cells but has little or no effect on normal cells (12). Therefore, TRAIL has been regarded as a potential drug for cancer therapy (12, 13). However, several kinds of cancer, including HCC, are not sensitive to soluble TRAIL treatment (14). HCC accounts for 80–90% of liver cancers and is one of the most prevalent carcinomas throughout the world, especially in Africa and Asia. Thus, it is worthwhile to find a new strategy to overcome the resistance of HCC cells to TRAIL-induced apoptosis.

Considering that Notch1 signaling up-regulates p53 and induces apoptosis of HCC cells and that there are no reports to date that address the relationship between Notch1 signaling and TRAIL-induced apoptosis, in this study, we investigated whether and how Notch1 signaling could sensitize HCC cells to TRAIL-induced apoptosis. We demonstrate that Notch1 signaling up-regulates p53 expression by inhibiting proteasome degradation via, at least in part, suppressing the phosphatidylinositol 3-kinase/Akt/Hdm2 pathway. In addition, we here report that Notch1 signaling enhances DR5 (death receptor 5) expression in a p53-dependent manner, and DR5 contributes, at least in part, to the enhancement of TRAIL-induced apoptosis by Notch1 signaling. Accordingly, Notch1 signaling sensitizes HCC cells to TRAIL-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cells, Reagents, and Antibodies**—HCC cells HepG2 and Hep3B were obtained from the ATCC, except for SMMC7721 cells (6). Human TRAIL was purchased from PeproTech (Rocky Hill, NJ). MG-132 and γ-secretase inhibitor X (γ-SIX) were purchased from Calbiochem; wortmannin and Delta4 were purchased from Sigma. Primary antibodies against Notch1, p53, Dcr1, Dcr2, DR4, DR5, and Hes1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-ac- tin and anti-FLAG M2 antibodies were from Sigma; antibodies against Ser473 phosphorylated Akt, Hdm2, and Ser166-phosphorylated Hdm2 were from Cell Signaling Technology (Beverly, MA).

**Expression Vector Construction and Cell Transfection**—The cDNA encoding a constitutively active form of Notch1, consisting of the intracellular domain (base pairs 5308–7665), was amplified by reverse transcription-PCR from SMMC7721 cells: forward primer, 5′-CCCTCAGAGATGCCTGAGGGCCTC- AAGTGCTCAGG-3′; reverse primer, 5′-CAAGGCTTCC- TTGAAGGGCTTCCGGAATGGCGG-3′. Notch1 intracellular domain was cloned into pcDNA3.1/FLAG(−)-B vector to generate FLAG-tagged expression vectors of the Notch1 intracellular domain (ICN).

ICN or pBFLAG mock vectors were transfected into HCC cells using JetPEI reagent (Polyplus), as described (15), and the transfected HCC cells were screened under 800 μg/ml G418 (Calbiochem) for 3–5 weeks. HCC cell clones of stably transfected cells were obtained by the limited dilution method, and their expression of ICN was confirmed by Western blot analysis.

**RNA Interference**—The p53-specific siRNA (si-p53, 5′-GAC-TCCAGTGTAATCTACTT-3′) and scrambled control (si-Non, 5′-UUCCUGAGCGUCAGGTUTT) were synthesized by GeneChem (Shanghai, China). Human DR5 siRNA was from Santa Cruz Biotechnology. siRNA was delivered into HCC cells using JetPEI reagent (Polyplus).

**Apoptosis Assay**—After treatment with TRAIL, HCC cells with or without ICN transfection were washed, resuspended in the staining buffer, and examined with the Vybrant apoptosis assay kit (Invitrogen) according to the manufacturer’s instructions (6). Stained cells were detected by FACS (FACScalibur; BD Biosciences). The Annexin V-positive and propidium iodide-negative cells were regarded as apoptotic cells.

**Western Blot Analysis**—Protein concentration was measured by the BCA protein assay reagent kit (Pierce). Samples containing equal amounts of protein (50 μg) were resolved by 10% SDS-PAGE and subjected to Western blot analysis (16). Blots were probed with the indicated primary antibodies with appropriate horseradish peroxidase-conjugated antibodies as secondary antibodies (Cell Signaling Technology). Relevant protein bands were visualized by using SuperSignal West Femto chemiluminescent reagents (Pierce).

**Preparation and Culture of Human Primary Hepatocellular Carcinoma Cells**—Fresh liver cancer tissues were obtained from the HCC patients at the Department of Surgery of Changzheng Hospital. Human primary hepatocellular carcinoma cells were prepared as described (17) with minor modification. Briefly, the liver cancer tissue was scraped into small pieces (1–1.5 mm3 in size) and then digested with collagenase (type IV, 0.1%; Amresco), hyaluronidase (type V, 0.01%; Amresco), and DNase (type I, 0.002%; Amresco) for 4 h. The single cell suspension was further isolated by Percoll gradient centrifugation (GE Healthcare) and cultured in RPMI 1640 plus 10% fetal calf serum with or without stimulation of human Delta4 (0.5 μg/ml) for 12 h.

**Statistical Analysis**—All data were presented as means ± S.E. p values were calculated using Student’s t test to compare results, and a value of p < 0.05 was considered as statistically significant.

**RESULTS**

Notch1 Signaling Up-regulates p53 Expression in HCC Cells by Suppressing the Proteasome Pathway—Our previous results have shown that Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis (6). Here we found that Notch1 was highly expressed in HepG2 and SMMC7721 HCC cells, whereas the expression of Notch2, Notch3, and Notch4 was weak. The Notch ligand, Delta4, was weakly expressed in both cells; oth-
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**Suppression of the Akt/Hdm2 Pathway in HCC Cells by Notch1 Signaling Contributes to the Inhibition of p53 Protein Degradation—Multiple pathways are involved in the regulation of p53 expression, among which the Akt/Mdm2 pathway has been shown to be important. Akt/Mdm2 signaling could phosphorylate p53, promote p53 proteasome degradation, and thus down-regulate p53 expression (18, 19). To investigate whether Akt was involved in the regulation of p53 in HCC cells by Notch1 signaling, we first detected the total expression and phosphorylation of Akt in SMMC7721 and HepG2 cells transfected with ICN. As shown in Fig. 2A, overexpression of ICN inhibited both the total level and the phosphorylation level of Akt in SMMC7721 and HepG2 cells. On the other hand, γ-SiX treatment enhanced the total level and the phosphorylation of Akt (Fig. 2B). We further pretreated HepG2 cells with the specific phosphatidylinositol 3-kinase inhibitor, wortmannin, and then treated these HCC cells with γ-SiX. As shown in Fig. 2C, γ-SiX-mediated down-regulation of p53 was partially reversed by Akt inhibition. Together, these data indicated that suppression of Akt pathway contributed to the up-regulation of p53 by Notch1 signaling.

Hdm2 (human homologue of Mdm2) could bind to and ubiquitinate p53 and down-regulate p53 protein expression by promoting proteasome degradation (8). We then wondered whether the Hdm2 pathway was involved in the Notch1 signaling-mediated up-regulation of p53. To address this question, we transfected a domain-negative vector encoding a mutant form of Hdm2 (Hdm2.S166A/S186A), as described previously (20), into HepG2 cells. As shown in Fig. 2D, γ-SiX-mediated down-regulation of p53 was also partially reversed by Hdm2.S166A/S186A transfection, indicating that inhibition of the Hdm2 pathway was also involved in the up-regulation of p53 by Notch1 signaling.

Akt-mediated phosphorylation of Hdm2 (human homologue of Mdm2) was observed in MCF-7 cells (21). Here we found that when cells were pretreated with wortmannin to...
inhibit Akt activation, both up-regulation of Hdm2 phosphorylation and down-regulation of p53 mediated by γ-SIX were reversed in HepG2 cells (Fig. 2E). The data indicated that suppression of the Akt/Hdm2 pathway was responsible for Notch1 signaling-mediated up-regulation of p53 in HCC cells via inhibition of p53 protein degradation.

Notch1 Signaling Sensitizes HCC Cells to TRAIL-induced Apoptosis through Up-regulation of p53—Many studies have shown that increase of p53 protein expression can sensitize TRAIL-induced apoptosis (22, 23). Previously we have also demonstrated that Notch1 signaling alone can induce apoptosis of HCC cells (6). The up-regulation of p53 by Notch1 signaling alone (Fig. 1A) inspired us to investigate whether Notch1 signaling can sensitize HCC to TRAIL-induced apoptosis and, if so, what the role of p53 is in the process. First, we examined the sensitivities of SMMC7721 and HepG2 cells to TRAIL-induced apoptosis by treating them with different concentrations (12.5, 25, 50, and 100 ng/ml) of TRAIL. Most of the TRAIL-sensitive cancer cells could be induced to apoptosis when less than 20 ng/ml TRAIL was used; however, as illustrated in Fig. 3A, only a high concentration (>50 ng/ml) of TRAIL could induce significant apoptosis of SMMC7721 and HepG2 cells (over 15%), indicating that SMMC7721 and HepG2 cells were relatively resistant to TRAIL-induced apoptosis at the concentrations we used.

Then we observed the apoptosis of cell clones stably expressing ICN (HepG2-ICN, HepG2-mock, SMMC7721-ICN, and SMMC7721-mock). The expression efficiency of ICN and p53 expression level was examined in HepG2 and SMMC7721 clones stably expressing ICN (Fig. S4, A and B). As shown in Fig. 3, B and C, overexpression of ICN alone could induce HCC apoptosis, but the induction effect was weak (6–8% versus 3–4% in mock cells). With the increase of TRAIL concentration, HepG2-ICN and SMMC7721-ICN cells exhibited significant apoptosis at a much lower concentration (12.5 ng/ml for HepG2 cells and 25 ng/ml for SMMC7721 cells) and became more sensitive to TRAIL-induced apoptosis at a much higher concentration. These findings indicated that Notch1 signaling sensitizes HCC cells to TRAIL-induced apoptosis.
To investigate the role of p53 in Notch1 signaling-enhanced apoptosis induction by TRAIL in HCC cells, we silenced p53 expression in HCC cells with specific siRNA for p53 (si-p53) and then observed the apoptotic response of p53-silenced HepG2 cells to TRAIL. We found that the sensitivity to TRAIL-induced apoptosis was decreased in p53-silenced HepG2 cells (Fig. 4A, \( p < 0.05 \)), and overexpression of ICN could not enhance TRAIL-induced apoptosis in p53-silenced HepG2 cells (Fig. 4A). Also, ICN overexpression lost the ability to enhance TRAIL-induced apoptosis in p53-defective Hep3B cells (Fig. 4B), further confirming the p53-dependent enhancement of TRAIL-induced apoptosis in HCC cells by Notch1 signaling. Together, the data suggested that Notch1 signaling sensitizes HCC cells to TRAIL-induced apoptosis through up-regulation of p53.

Notch1 Signaling Increases DR5 Expression in HCC Cells in a p53-dependent Manner, Leading to Sensitization of HCC Cells to TRAIL-induced Apoptosis—In addition to the role of p53, we went further to investigate whether other molecular mechanisms underlying the sensitization effect of Notch1 signaling to TRAIL-induced apoptosis may be involved. TRAIL transduces apoptotic signal via its receptors on the target cells. At least five receptors have been characterized, among which DR4 and DR5 are capable of transducing the apoptosis signal, whereas the other three (DcR1, DcR2, and OPG) have no or a short cytoplasmic tail and serve as decoy receptors to block TRAIL-mediated apoptosis (24, 25). We showed that DcR1 expression was detectable in HepG2 cells but not in SMMC7721 cells. DcR2, DR4, and DR5 were expressed in both SMMC7721 and HepG2 cells (Fig. 5A). When cells were transfected with ICN, DR5 expression was significantly increased in HepG2 cells and, to a lesser extent, in SMMC7721 cells (Fig. 5B). These results demonstrate that Notch1 signaling promotes DR5 expression in HCC cells, outlining molecular mechanism for the increased sensitivity of HCC cells to TRAIL-induced apoptosis mediated by Notch1 signaling. We further evaluated the role of DR5 in the ICN-mediated sensitization of HCC cells to TRAIL-induced apoptosis. As shown in Fig. 5D, silencing of DR5 expression could partially eliminate the enhancement in TRAIL-induced apoptosis by Notch1 signaling, suggesting that DR5

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**FIGURE 3.** ICN overexpression sensitizes HCC cells to TRAIL-induced apoptosis. SMMC7721 and HepG2 cells (A), ICN-overexpressing stable transfectant of SMMC7721 cells (SMMC7721-ICN) (B), and ICN-overexpressing stable transfectant of HepG2 cells (HepG2-ICN) (C) were treated with the indicated concentration of TRAIL for 24 h. Cells were then stained with Annexin V/propidium iodide for analysis of cell apoptosis by FACS. Annexin V-positive and propidium iodide-negative cells were regarded as apoptotic cells. **, \( p < 0.01 \) versus mock control. Data show means ± S.E. of triplicates from one experiment representative of three independent experiments.

**FIGURE 4.** ICN-mediated enhancement of TRAIL-induced apoptosis is dependent on up-regulation of p53 in HCC cells. A, HepG2 cells were transfected with siRNA specific for p53 (si-p53) or si-Non for 24 h, transfected with ICN or mock vector for 24 h, and then treated with the indicated concentration of TRAIL for 24 h. HepG2 cells transfected with ICN or mock vector for 24 h and then treated with the indicated concentration of TRAIL for 24 h. Cellular apoptosis was analyzed by FACS. **, \( p < 0.05 \) versus mock + si-Non control. Data show means ± S.E. of triplicates from one experiment representative of three independent experiments.
was involved in the process, and other mechanisms might also be involved.

It has been reported that DR5 is a downstream target of p53 (26). However, the induction of DR5 via p53-independent mechanisms has also been reported (27). So what is the role of p53 up-regulation in the increased DR5 expression in HCC cells mediated by Notch1 signaling? Was DR5 up-regulation p53-dependent or p53-independent? To address these questions, we first silenced p53 expression in SMMC7721 and HepG2 cells and then transfected these p53-silenced HCC cells with ICN. As shown in Fig. 6A and B, ICN overexpression-induced increase of DR5 expression in HCC cells was nearly completely abrogated in p53-silenced cells. In addition, overexpression of ICN could not up-regulate DR5 expression in p53-defective Hep3B cells (Fig. 6C). These findings suggested that Notch1 signaling sensitizes HCC cells to TRAIL-induced apoptosis through, at least in part, up-regulating DR5 expression in a p53-dependent manner.

**DISCUSSION**

Notch1 signaling has been found to be able to inhibit growth of some kinds of tumor cells, including HCC cells, small lung cancer cells, and HPV-positive cervical cancer cells (6, 28, 29). Because of killing a broad range of tumor cells without showing the deleterious side effects to normal cells, TRAIL is regarded as a unique agent for cancer treatment; however, HCC is resistant to soluble TRAIL treatment (14). Therefore, some efforts have been made to sensitize TRAIL-resistant HCC cells to the cytotoxicity of TRAIL, such as pretreatment of HCC with interferon-α and chemotherapeutic agents (30). Our present study for the first time demonstrates that Notch1 signaling can significantly sensitize HCC to TRAIL-induced apoptosis by up-regulating p53 expression and p53-dependent DR5 expression in HCC cells.

Notch1 signaling-induced up-regulation of p53 in HCC cells is due to the inhibition of the Akt/Hdm2 pathway by Notch1 signaling. p53 is one of the most important tumor suppressor genes in human cancer (31), and its protein level was mostly regulated by stabilization mediated with post-translation (32). Our result showed that inhibition of the Akt/Hdm2 pathway by γ-secretase inhibitor X could down-regulate p53 expression, but MG132, a specific proteasome inhibitor, could almost fully reverse such down-regulation of p53 expression, confirming that Notch1 signaling up-regulates p53 by enhancing p53 stability via suppression of p53 proteasome degradation. p53 expression is regulated by multiple signal pathways. For example, the reactive oxygen species can activate p38 kinase, which in turn promotes the activation of p53, resulting in induction of HepG2 apoptosis (33); JNK could phosphorylate p53 and suppress ubiquitin-mediated p53 protein degradation, thus stabilizing p53 protein (34). Recent studies demonstrate that activated Akt could phosphorylate Mdm2 at Ser166 and Ser186, resulting in a decrease in p53 expression (18). Our data showed that inhibition of the Akt/Hdm2 pathway could partially reverse γ-SiX-induced down-regulation of p53, suggest-
ing that suppression of the Akt/Hdm2 signal pathway by Notch1 signaling was responsible for up-regulation of p53 in HCC cells and also indicating that other signals might be involved in Notch1 signaling-induced up-regulation of p53. In our previous study, Notch1 signaling could promote the phosphorylation of JNK in HCC cells (6). Considering that JNK is able to up-regulate p53 (34), enhancement of JNK phosphorylation by Notch1 signaling might also contribute to the up-regulation of p53, and this hypothesis needs to be investigated in further study.

TRAIL-triggered apoptosis is determined by binding to and activating death receptors (DR4 and DR5), which belong to the tumor necrosis factor receptor gene superfamily. They are located at the cell surface and become trimerized through binding to their ligand TRAIL and then transmit apoptosis signals by rapid activation of caspase cascades (35). Numerous researchers have shown convincing data that TRAIL-induced apoptosis can be augmented by up-regulating the expression level of TRAIL receptors (i.e. DR4 and DR5) (36). Combined use of TRAIL and interferon-α or cisplatin has been found to induce more potent apoptosis of certain human hepatoma cells through DR5 or DR4 up-regulation (25, 37). Our data also extended such phenomena as Notch1 signaling increasing DR5 expression with enhancement of TRAIL-induced apoptosis in HCC cells. Several studies have shown that all four TRAIL receptors are regulated by p53 (38). Among them, DR5 is the first whose transcription has been shown to be directly activated via p53 through an intronic sequence-specific p53BS (26). Recent studies have shown that p53 also directly regulates the expression of DR4, DcR1, and DcR2 (39, 40). In our study, up-regulation of DR5 by Notch1 signaling did not exist in p53-silenced or p53-defective HCC cells, confirming that the up-regulation of DR5 by Notch1 signaling in HCC cells was p53-dependent. Furthermore, DR5 knockdown partially eliminated the enhancement of TRAIL-induced apoptosis by ICN, suggesting that DR5 contributes, at least in part, to the Notch1 signaling-mediated sensitization of TRAIL-induced apoptosis.

We here provide a possible mechanistic model by which Notch1 signaling sensitizes TRAIL-induced apoptosis by ICN, suggesting that DR5 contributes, at least in part, to the Notch1 signaling-enhanced apoptosis induction by TRAIL in HCC cells. The model depicts the involvement of Notch1 signaling in extrinsic and intrinsic apoptotic pathway. Notch1 activation suppressed the Akt/Hdm2 pathway, leading to up-regulation of p53 via suppression of p53 proteasome degradation and p53-dependent up-regulation of DR5, resulting in cellular apoptosis.
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through the induction of DR5; in addition, p53 can also induce the intrinsic (mitochondrial) pathway of cellular apoptosis by targeting several p53-regulated apoptotic target genes. Together, we for the first time have provided the evidence that Notch1 signaling sensitizes HCC cells to TRAIL-induced apoptosis. Therefore, the combined use of Notch1 signaling and TRAIL may be effective in the treatment of TRAIL-resistant HCC.

Acknowledgments—We thank Dr. Han C (Beth Israel Deaconess Medical Center, Harvard Medical School) for kindly providing the Hdm2 (S166A/S186A) mutant. We also thank Yan Li and Jin Mei for excellent technical assistance.

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