RESEARCH ARTICLE

A Functional SNP in the MDM2 Promoter Mediates E2F1 Affinity to Modulate Cyclin D1 Expression in Tumor Cell Proliferation

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Abstract

Background: The MDM2 oncogene, a negative regulator of p53, has a functional polymorphism in the promoter region (SNP309) that is associated with multiple kinds of cancers including non-melanoma skin cancer. SNP309 has been shown to associate with accelerated tumor formation by increasing the affinity of the transcriptional activator Sp1. It remains unknown whether there are other factors involved in the regulation of MDM2 transcription through a trans-regulatory mechanism. Methods: In this study, SNP309 was verified to be associated with overexpression of MDM2 in tumor cells. Bioinformatics predicts that the T to G substitution at SNP309 generates a stronger E2F1 binding site, which was confirmed by ChIP and luciferase assays. Results: E2F1 knockdown downregulates the expression of MDM2, which confirms that E2F1 is a functional upstream regulator. Furthermore, tumor cells with the GG genotype exhibited a higher proliferation rate than TT, correlating with cyclin D1 expression. E2F1 depletion significantly inhibits the proliferation capacity and downregulates cyclin D1 expression, especially in GG genotype skin fibroblasts. Notably, E2F1 siRNA effects could be rescued by cyclin D1 overexpression. Conclusion: Taken together, a novel modulator E2F1 was identified as regulating MDM2 expression dependent on SNP309 and further mediates cyclin D1 expression and tumor cell proliferation. E2F1 might act as an important factor for SNP309 serving as a rate-limiting event in carcinogenesis.

Keywords: MDM2 - cyclin D1 - E2F1 - carcinogenesis

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Introduction

Mouse double-minute 2 homologue (MDM2) is a key negative regulator of the tumor suppressor p53 that initiates cell cycle arrest and apoptosis upon cellular stresses (Chen et al., 2014; Song et al., 2014). MDM2 directly binds to and inhibits p53 pathway by regulating its location, stability and activity (Michael and Oren, 2003). The mice with reduced MDM2 expression showed increased apoptosis in both epithelial and lymphocytes cells (Mendrysa et al., 2003). In humans, MDM2 overexpression is associated with accelerated cancer progression in multiple cancers including glioblastomas, bladder cancer and breast cancer (Schiebe et al., 2000; Hori et al., 2002; Burton et al., 2002; Tuna et al., 2003; Hitzenbichler et al., 2013; Xiong et al., 2013). In a subset of tumors, overexpression of MDM2 can substitute for inactivating p53 by mutation (Oliner et al., 1992; Leach et al., 1993). Jone et al. reported that Mdm2-overexpressing mice developed spontaneous tumors in a lifetime (Jones et al., 1995). Antagonizing MDM2 to inactivate p53 pathway might offer a new therapeutic strategy for tumorgenesis (Vassilev, 2007; Shangary and Wang, 2009).

Except for the alterations of expression level, naturally occurring polymorphic genetic variants in MDM2 was found in both hereditary and sporadic cancers and several studies revealed that SNP309 could positively impact tumor formation (Onat et al., 2006; Shinohara et al., 2009; Zawlik et al., 2009; Azmi et al., 2010; Cheng et al., 2012; Zhao et al., 2012). The occurrence of single nucleotide polymorphisms (SNP) is tightly associated with carcinogenesis and susceptibility (Luo et al., 2014; Zeichner et al., 2014; Guan et al., 2014). Epidemiological analysis discovered that carriers of the MDM2 SNP309 G allele had an elevated risk of non-melanoma skin cancer (Almquist et al., 2011). Mechanistically, a single nucleotide polymorphism (SNP309) in MDM2 promoter was shown to increase the transcriptional activator Sp1 binding affinity, resulting in prohibited p53 pathway and heightened transcription (Bond et al., 2004). In view of the DNA binding complexity, we tested the possibility that the binding affinity of other transcription factors might be also affected by SNP309 polymorphism. Bioinformatics predicts that a possible E2F1 binding site was generated after T/G substitution at SNP309. Moreover, the molecular mechanism underlying MDM2 polymorphism in skin

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carcinogenesis remains unclear. Therefore, E2F1 mediated alteration of MDM2 promoter transcriptional activity in skin cancer cells was explored.

E2F1 functions as a coactivator to promote cell proliferation or growth in gastric, breast and liver cancers (Louie et al., 2004; Stender et al., 2007; Frietze et al., 2008; Li et al., 2008; Petrocca et al., 2008). Deregulated cell proliferation together with the obligate suppression of apoptosis is the hallmark of various carcers (Evan and Vousden, 2001). Consistent with our hypothesis, MDM2 positively augments cell proliferation in both tumor cells and normal human fibroblasts (Blaydes and Wynford-Thomas, 1998; Zhang et al., 2003; Francoz et al., 2006). Furthermore, previous studies demonstrated that E2F1 transcriptional activity and protein stability were regulated by oncoprotein MDM2 (Martin et al., 1995; Kowalik et al., 1998; Zhang et al., 2005; Ambrosini et al., 2007) and that there was a crosstalk between E2F1/Rb and MDM2/p53 pathways in cancer cell proliferation and apoptosis (Zhang et al., 2003). However, it is reasonable to postulate that E2F1 might reversely regulate MDM2 activity at transcriptional level dependent on SNP309.

In this study, E2F1 binding site is verified and SNP309 enhances the binding affinity of E2F1 in MDM2 promoter. Functional analysis suggests that E2F1 mediated MDM2 upregulation with T to G substitution leads to elevated Cyclin D1 expression and cell proliferation rate. Targeting of these regulatory events might provide potent therapeutic consequences.

Materials and Methods

Cell culture and patients

The uses cell lines were originally purchased from American Type Culture Collection (ATCC) and mainly grown in RPMI 1640 containing 10% serum according to the instructions. The MDM2 promoter was analyzed for sequence variation by PCR amplification and subsequent sequencing in these human cell lines and established fibroblasts from patients. The primers were: forward-CGGGAGTT CAGGGTAAAGGT, reverse-AGCAAGTCGGTGCTTACCTG (Bond et al., 2004). The randomly selected skin cancer patients were genotyped with the above primers. Written informed consent was obtained from study participants. Three patients' normal skin samples adjacent skin cancer tissues for each genotype were collected during surgery. Then the cells were trypsinized to single cells and plated in dishes using DMEM supplemented with 10% FBS.

Western Blot Analysis

Cell lysates from different treatment groups were prepared by boiling cells in SDS. Then the samples were electrophoresed on 10% Tris–glycine gels and then transferred onto nitrocellulose membranes. After blocking with 5% non-fat dry milk, the membranes were incubated with primary antibodies against MDM2 (1:200; Santa Cruz), Cyclin D1 (1:300; Santa Cruz), E2F1 (1:500, Santa Cruz), and β-actin (1:10,000; Abcam) at 4°C overnight or 3 h at room temperature, followed by incubation with a peroxidase-conjugated secondary antibody (1:1000; Santa Cruz). Then it was developed using an enhanced chemi-luminescence kit (Pierce, Appleton, WI).

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as previously described (Bond et al., 2004). Briefly, proteins were cross-linked to DNA in 1% formaldehyde. Then the cells were lysated and sonicated to proper DNA fragments (200-500 bp). 4 ug antibodies were added and incubated overnight. Protein A/G Plus beads (Santa Cruz) were used, following extensive washing and reverse-crosslink. Then DNA was isolated using the QIAquick PCR purification kit (Qiagen). Ten percent of purified DNA was analyzed by PCR. Non-immunoprecipitated DNA served as input samples.

Luciferase Assay

The MDM2 promoter-luciferase reporter plasmids containing either the wild-type sequence or the SNP309 sequence, as shown in Figure 1D, were constructed into pGL3 luciferase reporter plasmid (Promega) (Bond et al. 2004). H460 cells were plated in 6-well plates at 50 % confluency and co-transfected with T/T or G/G reporter plasmids, pRL-TK (Renilla, Promega), and pcDNA3-E2F1 or pcDNA3 using lipofectimine 2000 reagents (Invitrogen). Firefly and Renilla luciferase activities were determined 48 h post-transfection using the Dual Luciferase Reporter Assay System (Promega). Each group

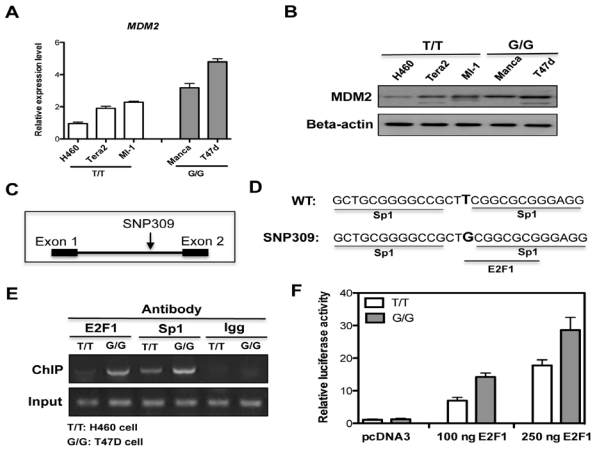

Figure 1. SNP 309 in MDM2 Promoter Alters the Affinity of the Transcriptional Factor E2F1. (A) The relative expression level of MDM2 in H460, Tera2, MI-1 cells (T/T genotype) and Manca , T47d cells (G/G genotype). (B) The indicated cells were fractioned and subjected to western blot analysis of MDM2 and beta-actin level. (C) The schematic diagram of SNP309 in DNA sequence of MDM2. (D) The DNA sequences around SNP309, which contains thymine (T) to guanine (G) substitution. Computer analysis predicts two Sp1 binding sites and a newly generated E2F1 binding site. (E) ChIP assay was performed to analyze the E2F1, Sp1 and Igg enrichment in SNP309 contained elements in H460 cells and T47D cells, using anti-E2F, anti-Sp1, normal Igg, relatively. (F) The relative luciferase levels are measured. pGL3 luciferase reporter plasmids containing either SNP309 sequence (G/G) or wild-type sequence (T/T) were cotransfected with pcDNA3 or indicated amount of pcDNA3-E2F1 expression plasmids. Each value is the average of at least three independent experiments and the error bars represent the standard deviations

was analyzed in duplicate, and at least three independent experiments were performed.

RNA Extraction and q-PCR

Total RNA was extracted from the cells with different treatment using RNeasy (Qiagen) and cDNA was synthesized using the reverse transcription reaction reagents (Superscript III, Invitrogen, CA). Relative gene expression level was determined using SYBR Green PCR Master Mix with a Bio-Rad thermocycler and SYBR Green detection system (Bio-Rad Laboratories). The relative expression levels of genes were normalized to GAPDH. The $2^{-\Delta\Delta Ct}$ methods were used to quantify the relative gene expression. Primer sequences were: MDM2 (forward, TCGTCGGGTGAGGGTACTG; reverse, AACCACTTCTTGGAACCAGGT), Cyclin D1 (forward, GCTGCGAAGTGGAAACCATC; reverse, CCTCCTTCTGCACACATTTGAA), E2F1 (forward, ACGCTATGAGACCTCACTGAA; reverse, TCCTGGGTCAACCCCTCAAG), GAPDH (forward, TGTGGGCATCAATGGATTTGG; reverse, ACACCATGTATTCCGGGTCAAT).

Cell Proliferation Assay

Cell proliferation was determined by microculture tetrazolium (MTT) assays as previously described (Liu et al., 2013). Identical number of cells were seeded in 24-well plates and transfected with indicated plasmids. After transfection for indicated time intervals, cells were incubated with MTT in culture medium for an additional 4-6 h. The cell proliferation was determined spectrophotometrically at 570 nm (OD570). The mean values of three wells were calculated for final OD570 value.

Gene Silencing with siRNA

E2F1 siRNA (5'-GUCACGCUAUGAGACCUCA-3') targeted to E2F1 mRNA (Jung et al., 2007) was used to reduce E2F1 expression. Control siRNA (5'-GUACAUUUCUCAGACCUCC-3') has no known target in mammalian genomes. Chemically synthesized siRNA duplex (GeneChem, China) was transfected into cells at about 50% confluencey using oligofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Prediction and Statistical Analysis

The transcription factor binding site prediction in SNP309 containing elements were performed in the website (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). All results in this study are expressed as the means ± standard deviation. Student's t-test was employed to determine the statistical difference between different groups. All results were repeated at least for three times. The data were considered significant if $P < 0.05$.

Results

SNP309 in MDM2 promoter alters the binding affinity of the transcriptional factor E2F1

Previously, a single nucleotide polymorphism (SNP309) was identified in MDM2 promoter to increase the affinity of transcriptional activator Sp1 (Bond et al., 2004). To verify the correlation between SNP309 and increased levels of MDM2, the mRNA and protein levels of MDM2 were examined in three tumor-derived cell lines wild-type for SNP309 (T/T) and two tumor-derived cell lines homozygous for SNP309 (G/G). Generally, MDM2 showed higher expression level in G/G genotype cell lines than T/T genotype at both transcriptional and translational levels (Figure 1A, B). SNP309 was found at the 309th nucleotide in the first intron (Figure 1C) with relatively high frequency and this genetic variation altered the Sp1 binding affinity and p53 activity (Bond et al., 2004). Inspired by this finding, we were wondering whether the binding affinity of other factors was affected by SNP309. Then we analyzed the SNP309 containing region in MDM2 promoter using a computer algorithm and revealed several Sp1 binding sites and a newly generated putative E2F1 binding site in G/G genotype (Figure 1D).

In order to characterize this potential E2F1 binding site predicted by computer analysis and to explore the functional consequences of SNP309, chromatin immunoprecipitation (ChIP) and luciferase assay were performed. Cell lysates were prepared from H460 cells, homozygous for SNP309 (T/T), and T47D cells, homozygous for SNP309 (G/G). Immunoprecipitations were carried out using antibodies against either E2F1 or Sp1, and normal Igg served as a negative control. The presence of E2F1 or Sp1 in MDM2 promoter was determined for using PCR. As shown in Figure 1E, the MDM2 promoter was detected in the ChIP using the Sp1 and E2F1 antibody but not using the Igg control and Sp1 showed stronger binding activity in G/G genotype cells. Importantly, the binding activity in T/T cells was close to the Igg control while the affinity of E2F1 in MDM2 promoter was significantly higher in G/G cells, suggesting that the T to G variation generates a new E2F1 binding site. These data demonstrate that E2F1 could efficiently bind to the MDM2 promoter homozygous for SNP309 G/G genotype.

To investigate a possible role of E2F1 in the transactivation of MDM2 promoter containing SNP309, H460 cells were transfected with gradient Sp1 expression vector and equal luciferase reporter plasmid driven by the MDM2 promoter with either T/T or G/G genotype. Figure 1E showed that E2F1 strongly stimulated elevation of MDM2 promoter driven luciferase activity, suggesting thatE2F1 can bind to the SNP309 containing region of the MDM2 promoter and activate its transcription. Notably, the reporter with SNP309 G/G genotype consistently showed higher E2F1-induced luciferase expression comparing with the wild-type sequence in the reporter plasmid. Together, these date suggest that, similar to Sp1, E2F1 can bind to the MDM2 promoter and activate transcription and that SNP309 presence stimulates the binding affinity of E2F1 to MDM2 promoter.

E2F1 regulates MDM2 expression in tumor cells

Previous findings support an association of SNP309 with the increased levels of MDM2, and the above results reveals a transactivation of MDM2 promoter by E2F1. It leads us to test whether the E2F1 is indeed

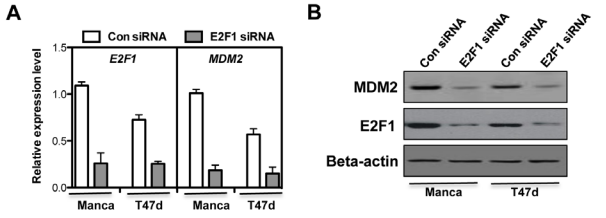

Figure 2. E2F1 Associates with the Overexpression of MDM2 in Tumor Cells. (A) The relative expression levels of E2F1 and MDM2 were determined by q-PCR in control (Con) siRNA or E2F1 siRNA transfected Manca and T47d cells after transfection for 48 h. (B) Western blot anlysis of MDM2, E2F1 and beta-actin protein levels in cells depicted in (A)

responsible for MDM2 upregulation in cells homozygous for G/G genotype. E2F1 siRNAs specific to E2F1 RNA were applied to reduce endogenous E2F1 level and the resulting effect on endogenous MDM2 level was analyzed. E2F1 siRNA efficiently downregulated the mRNA and protein levels in two MDM2 highly expressed cell lines (Manca and T47d) when compared to E2F1 levels in cells transfected with a non-specific control siRNA (Figure 2A, B). As expected, reduction of E2F1 levels resulted in the downregulation of MDM2 at both mRNA and protein levels. It suggests that E2F1 inhibition can preferentially reduce the heighened levels of MDM2 in cells SNP309 (G/G), thereby supporting the hypothesis that the transcription factor E2F1 is indeed responsible for MDM2 overexpression in tumor cells homozygous for SNP309.

Cyclin D1 is responsible for E2F1-mediated cell proliferation in skin cells homozygous for SNP309

MDM2 SNP309 is highly associated with the risk of multiple cancers and accelerated age of onset of tumor (Bond et al., 2004; Dharel et al., 2006; Hu et al., 2007; Zhou et al., 2007; Willander et al., 2010; Dong et al., 2012; Olsson et al., 2013), while the correlation between SNP309 and the characteristics of tumor cells has not been fully elucidated. Bond et al reported that Sp1 reduction in tumor cells lead to the down regulation of its know target gene Cyclin D1 (Grinstein et al., 2002; Bond et al., 2004), a nuclear protein required for cell cycle progression and proliferation in human tumor cells and normal cells (Baldin et al., 1993; Lukas et al., 1994; Stacey, 2003). Thus, the cell proliferation rates of tumor cells with differential SNP309 genotype were measured by OD570. Interestingly, the proliferation of tumor cells homozygous for SNP309 G/G genotype (Manca and T47d) is significantly higher than T/T genotype cells (H460 and Tera2) (Figure 3A). Moreover, the expression of Cyclin D1, which could reflect the ability of cell proliferation, in SNP309 G/G genotype cells showed higher protein levels than wild-type cells (Figure 3B). It gives us a hint that SNP309 in MDM2 promoter might interfere tumor cell proliferation.

In view of the differential origin of the studied cell lines, it would be more reasonable to compare cell proliferation of the same cell type with distinct SNP309 genotypes. SNP309 in MDM2 promoter was associated with the risk of non-melanoma skin cancer (Almquist et al.,

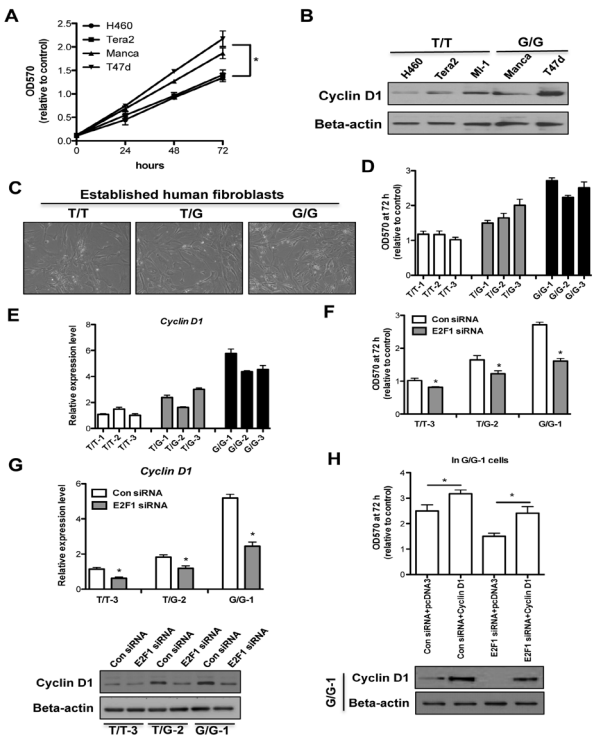

Figure 3. Cyclin D1 is Responsible for E2F1-mediated Cell Proliferation in Skin Cells Homozygous for SNP309. (A) Cell proliferation was examined at 24, 48 and 72 h in H460, Tera2, Manca and T47d cells. The OD570 values were considered as an indicator of cell proliferation. (B) Western blot analysis of Cyclin D1 protein level in T/T genotype cells (H460, Tera2 and MI-1) and G/G genotype cells (Manca and T47d). (C) Examples of established human fibroblasts with T/T, T/G and G/G genotypes were shown as bright fields. (D) The bar graph indicates the OD570 values of wide type T/T fibroblasts (T/T-1, -2, -3), heterozygous T/G mutant fibroblasts (T/G-1, -2, -3) and homozygous G/G mutant fibroblasts (G/G-1, -2, -3) after culturing for 72 h. (E) The relative expression level of Cyclin D1 in the cells referred in (D) was determined by q-PCR. (F) The OD570 value of T/T-3, T/G-2 and G/G-1 fibroblasts, which were transfected with control (Con) siRNA or E2F1 siRNA, was determined at 72 h. (G) The relative expression levels of Cyclin D1 in cells referred as (F) were examined by q-PCR and their protein levels were determined by western bolt analysis. Beta-actin served as a loading control. (H) The OD570 values of homozygous G/G-1 cells, which were transfected with con siRNA plus pcDNA3, con siRNA plus Cyclin D1, E2F1 siRNA plus pcDNA3 or E2F1 siRNA plus Cyclin D1, respectively, were examined at 72 h. Then these transfected cells were fractioned and subjected to western blot using anti-Cyclin D1 and anti-beta-actin antibodies

2011). Therefore, primary cultured human fibroblasts were established from skin cancer patients' dissected tissues, who were diagnosed as non-melanoma skin cancer with SNP309 T/T, T/G, and G/G genotypes (Figure 3C). Three independent cell lines were established for each genotype and cell proliferation rates were measured by OD570 values. Consistent with the observations in Figure 3A, the human fibroblasts with G/G genotype showed much higher cell proliferation rate than T/T genotype and T/G genotype skin cells displayed intermediate proliferation ability (Figure 3D). Importantly, the expression levels of Cyclin D1 in primary cultured fibroblasts that were determined by qPCR, showed similar patterns with OD570 values

between different genotypes (Figure 3E). Based on our findings that SNP309 G/G genotype was associated with MDM2 overexpression, which was modulated by E2F1 binding and regulation, the effect of E2F1 knockdown on cell proliferation of skin cells with different genotypes was tested. Three fibroblast cell lines with T/T, T/G, and G/G genotypes, respectively, were transfected with E2F1 siRNA. The result in Figure 3F showed that E2F1 inhibited cell proliferation in these three genotyped fibroblasts.

To further explore the correlation between the proliferation indicator Cyclin D1 and E2F1, Cyclin D1 expression was examined in E2F1 siRNA transfected fibroblasts. Cyclin D1 expression was downregulated by E2F1 siRNA at both mRNA and protein level (Figure 3G). Notably, the inhibitory effects of E2F1 siRAN on cell proliferation and Cyclin D1 expression were more significant in G/G genotype fibroblasts than cells with the other two genotypes (Figure 3F, G). To investigate whether Cyclin D1 is responsible for E2F1 mediated cell proliferation in human fibroblasts, Cyclin D1 expression vector were cotransfected with E2F1 siRNA or control siRNA. The results in Figure 3H showed that Cyclin D1 overexpression promoted cell proliferation while E2F1 knockdown suppressed it. More importantly, the inhibitory effect of E2F1 reduction on cell proliferation was rescued by Cyclin D1 overexpression (Figure 3H). Taken together, Cyclin D1 acts downstream of the E2F1-MDM2 regulatory cascade to regulate skin cell proliferation homozygous for SNP309.

Discussion

In recent years, the genetic variations which underlie the phenotypic differences in induviduals' susceptibility to cancer have been widely studied (Del et al., 1996; Fearon, 1997; Su et al., 2014). MDM2 overexpression and a single nucleotide (SNP309) are associated with occurrence of multiple kinds of cancers (Bond et al., 2004; Dharel et al., 2006; Almquist et al., 2011; Burton et al., 2002; Dong et al., 2012). The increase of Sp1 binding affinity by SNP309 in MDM2 promoter partially explains the overexpression of MDM2 in tumors, which leads to the attenuation of the p53 DNA damage response (Bond et al., 2004). In this study, the enhancement of Sp1 binding affinity by SNP309 was also observed (Figure 1). As a positive control, it proves the reliability of our findings. Whereas, Sp1 encoded protein acts as a general promoter activator, which is involved in many cellular processes, including differentiation, cell growth, apoptosis, and immune responses of normal and cancer cells (Frazer et al., 2009). There should be a specific factor involved in SNP309 binding affinity, which could mediate tumorgenesis.

To search for a specific transcription factor that was affected by SNP309, the restricted DNA sequences around SNP309 were analyzed by computer and a newly generated potential E2F1 binding site was found. The biochemical assays in cell culture systems demonstrate that SNP309 in MDM2 promoter strongly increases the binding affinity of E2F1, while its binding activity is very limited in wilt-type cells. Moreover, E2F1 could efficiently activate MDM2 promoter activity (Figure 1). Although we present here that T to G substitution generates a new E2F1 binding site, while E2F1 overexpression activates the wild-type MDM2 reporter activity (Figure 1F), suggesting that the reporter plasmid might contain other E2F1 binding sites, which could be activated by E2F1. Consistent with previous findings (Bond et al., 2004), SNP309 T to G substitution is associated with MDM2 overexpression, and heightened MDM2 expression was coordinately regulated by E2F1 (Figure 2) and Sp1. Previous studies revealed that MDM2 oncoprotein could stimulate or blocked E2F1 transcriptional activity (Martin et al., 1995; Kowalik et al., 1998), suggesting the complexity of the regulatory relationship between E2F1 and MDM2. Our results here, combining with these reports, demonstrate that targeting E2F1-MDM2 signaling cascade might provide a new insight for cancer therapy.

Numerous reports state that the heightened levels of MDM2, due to SNP309, result in accelerated tumor formation in humans (Hori et al., 2002; Zhou et al., 2007; Willander et al., 2010; Almquist et al., 2011; Zhao et al., 2012; Olsson et al., 2013). Whereas, the impact on tumor cell characteristics by MDM2 overexpression has not been fully elucidated. According to the experimental data presented in this study and previous report, MDM2 reduction elicited by E2F1 or Sp1 knockdown, accompanies with Cyclin D1 downregulation (Bond et al., 2004), which plays essential roles in tumor cell proliferation (Baldin et al., 1993; Stacey, 2003). Coincidently, the cell proliferation rates in distinct tumor cell lines or primary cultured human fibroblasts with different SNP309 genotypes were different (Figure 3). The G/G genotype cells showed higher MDM2 expression level as well as higher proliferation rate, and the E2F1-MDM2 cascade promoted cell proliferation is achieved by Cyclin D1 (Figure 3). These data suggest that SNP309 can impact tumorigenesis in humans through promoting cell proliferation through upregulation of Cyclin D1 expression. MDM2 is a key negative regulator of p53 and regulates the degradation of p53 in SNP309 (G/G) cells (Michael and Oren, 2003). p53 has been reported to inhibit Cyclin D1-dependent activity in growth suppression (Del et al., 1996). It is possible that SNP309-related MDM2 overexpression might result in p53 attenuation and Cyclin D1 upregulation simultaneously or sequentially, finally resulting the promotion of cell proliferation.

In summary, SNP309 variation, as a rate-limiting event in carcinogenesis, leads to the enhancement of E2F1 binding affinity. Then E2F1 directly activates MDM2 SNP309 (G/G) containg promoter, upregulates MDM2 expression, and accelerates cell growth through elevated Cyclin D1 expression, which could promote cell cycle progression (Baldin et al., 1993; Stacey, 2003). Therefore, activation of the Cyclin D1 pathway by SNP309 might affect cell growth related events and determine an individual's susceptibility to cancer. It might be a key initiating reason for accelerated carcinogenesis.

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