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RESEARCH PAPER

Metastasis-induction and apoptosis-protection by TWIST in gastric cancer cells

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Abstract TWIST, a basic helix-loop-helix transcription factor, has been recently reported to play an important role in tumorigenesis of human cancer through converting the early stage tumors into invasive malignancies. Upregulation of TWIST is often found in cancer patients, especially those with shorter survival period and poor response to chemotherapy. Here we studied the functions of TWIST on regulating migration rate, apoptosis, and gene expression in gastric cancer cells. TWIST expression is elevated in MGC-803 and HGC-27 cells that exhibit high invasive potential; whereas it is reduced in BGC-823 and SGC-7901 cells that possess relatively low invasive content. To evaluate functional consequences of TWIST induction, we examined the effect of TWIST on cell migration and apoptosis. Overexpression of TWIST in BGC-823 cells resulted in increased migration content and decreased sensitivity to the arsenic oxide-induced cell death. Moreover, small interference RNA-mediated TWIST ablation in MGC-803 and HGC-27 cells showed suppressed migration ability, increased induction of apoptosis in response to arsenic oxide, and elevated cell cycle arrest. Furthermore, we found a negative correlation between the TWIST level and p53 level, probably due to transcriptional regulation. Our results have identified TWIST as a critical regulator of gastric cancer cell proliferation and migration, suggesting a potential therapeutic approach to inhibit the growth and metastasis of gastric cancer through inactivation of TWIST.

Keywords TWIST · Epithelial mesenchymal transition · p53 · Apoptosis · Metastasis · Gastric cancer · Arsenic oxide

Abbreviations

bHLH	Basic helix-loop-helix
EMT	Epithelial mesenchymal transition
FCM	Flow cytometry
MET	Mesenchymal epithelial transition
PI	Propidium iodide

Introduction

TWIST belongs to the basic helix-loop-helix (bHLH) family of transcription factors that play a central role in cell type determination and differentiation in both vertebrates and invertebrates. In fly, TWIST determines the establishment of dorsoventral tissue pattern in the gastrulating embryo and the specification of mesodermal fates during cell differentiation [1]. In mouse, TWIST is crucial on the development of cephalic neural tube, cranial mesoderm, branchial arches, and limb buds [2]. Loss of one or two

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alleles of *TWIST* gene causes variable physical abnormalities and defects in growth and patterning [2]. In humans, *TWIST* gene deletion or depressed expression leads to Saethre-Chotzen syndrome, an autosomal dominant disease characterized clinically by facial dysmorphism, digit defects, and premature fusion of coronal sutures [3].

The bHLH proteins are defined by the basic domain that mediates specific DNA binding and the HLH domain that operates protein–protein interaction. Accordingly, the homo- or hetero-dimerized complexes of TWIST generate different DNA-binding properties. Depending on the protein partners in the dimers, these complexes play a significant impact on the expression of downstream genes, either as an activator or a repressor of transcription. For example, TWIST homodimer can recognize and bind to the E-box motif on the promoter of *E-cadherin* gene, repressing its expression [4]. Moreover, TWIST was validated recently to be involved in PI3K/AKT signal pathway by directly binding to the E-box of *AKT2,* consequently increasing AKT2 level [5]. The diverse effects of TWIST on the expression of different genes through the same type of interaction represent just one profile of the complexity of gene regulation network conducted by TWIST. Such a complexity is further exacerbated by the regulation of TWIST on its target genes' expression. For instance, interaction between TWIST and an E-box can be disrupted by the binding of TWIST with negative regulators such as Id-1, which antagonizes TWIST function by binding with TWIST, leading to the loss of its E-box binding ability [6].

Recent studies have implicated that TWIST is a potent oncogenetic factor due to its epithelial mesenchymal transition (EMT) stimulative, apoptosis-resistant and differentiation-inhibitive properties. Overexpression of TWIST is frequently found in a big variety of cancers and strikingly correlated to the metastasis of cancer and poor prognosis [7–10]. EMT is pivotal for the transformation of early stage tumor into invasive malignancy [11]. Cells undergoing EMT lose expression of epithelial markers such as E-cadherin, α-catenin and β-catenin, but acquire expression of mesenchymal components such as vimentin, fibronectin and N-cadherin [12], resulting in loss of cell polarity, cell-basement adhesion and cell–cell contact. As an EMT promoting factor, TWIST plays a central role in down-regulating E-cadherin and β-catenin, up-regulating vimentin and N-cadherin. TWIST overexpression inhibits mammary cell differentiation and this inhibition could be reversible by blocking TWIST expression [13]. Moreover, TWIST has also been demonstrated to inhibit apoptosis in mouse embryonic fibroblasts [14], nasopharyngeal carcinoma cells [15], and prostate cancer cells [16].

Gastric cancer is one of the most common malignancies which frequently metastatize from primary cancer site to liver or peritoneal membrane due to the dramatic cellular and molecular changes reminiscent of EMT [17]. TWIST has been found to be elevated in gastric cancer [18, 19]. However, the role of TWIST during the process of gastric cancer development is not well understood. In this report, we studied the correlation between TWIST level and the migration rate, the cell death response to chemotherapeutic drug, and the changes of p53 level in gastric cancer cells. We found that overexpression of TWIST in BGC-823 cells—a line expressing basal level of TWIST—resulted in increased migration ability, decreased apoptosis, up-regulation of mesenchymal markers, down-regulation of epithelial marker, and reduced level of p53. Reverse phenotypes were observed in siRNA-mediated TWIST-depleted MGC-803 and HGC-27 cells, which express high level of TWIST. Taken together, we have identified that TWIST was associated with resistance to apoptosis induced by arsenic oxide, presumably via a p53-dependent mechanism. Our findings suggest an underlying molecular mechanism responsible for the TWIST-mediated malignant transformation, cancer chemoresistance and suggest a target for overcoming chemodrug resistance in gastric cancer cells.

Results

Correlation between TWIST level and invasion ability

With intent to find gastric cell line with highly invasive capability and high TWIST level, we screened four cell lines (BGC-823, HGC-27, MGC-803, and SGC-7901). The well-differentiated SGC-7901 and the undifferentiated HGC-27 were derived from metastatic lymph nodes of gastric cancer [20, 21], while the poorly-differentiated MGC-803 and BGC-823 lines were originally obtained from gastric mucinous adenocarcinoma [22, 23]. Initially, we performed invasion assay to test the invasion ability of these cell lines. After 16 h of incubation, the transited and resided MGC-803 and HGC-27 cells were clearly increased compared with BGC-823 and SGC-7901 cells (Fig. 1a). The number of resided cells did not differ between HGC-27 and MGC-803 cells, with about 33 cells per field (Fig. 1b). These numbers are, as shown in Fig. 1b, significantly higher than either BGC-823 cells (about 6 cells/field, $P < 0.001$) or SGC-7901 cells (about 13 cells/field, $P < 0.001$). These results indicate that HGC-27 and MGC-803 cells display remarkably higher invasion ability than either BGC-823 or SGC-7901 cells.

We thought the diverse invasion ability of these gastric cancer cells might be linked to the different levels of TWIST. Indeed, TWIST protein in MGC-803 and HGC-27 cells was more abundant than in BGC-823 and SGC-7901 cells (Fig. 1c). In addition, we measured the cell surface markers to determine the variations of mesenchymal and

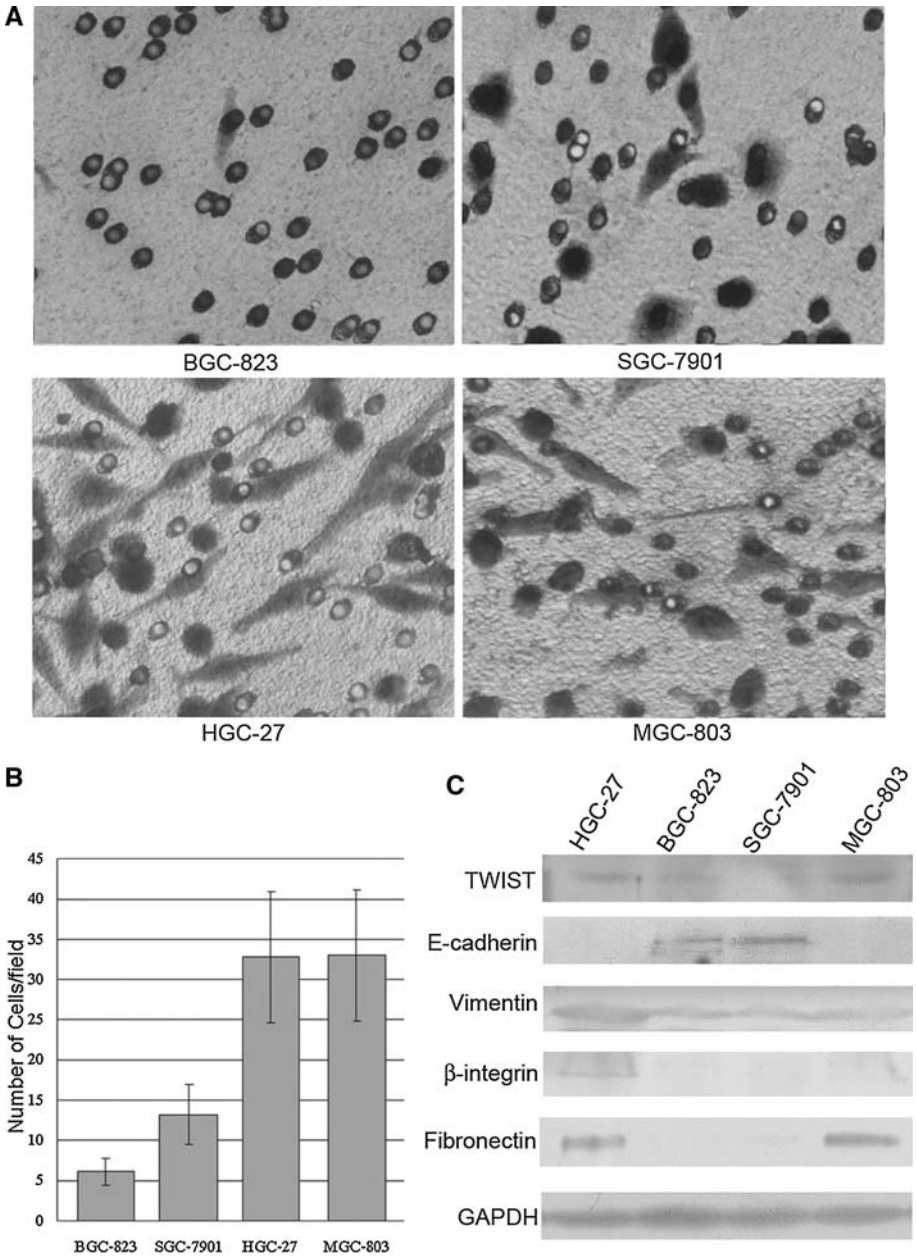

Fig. 1 Invasion ability and molecular character are different among four gastric cancer cell lines. **a** The invasive ability of the four gastric cancer cell lines assessed by Transwell assay. Images are representative of the migration of four gastric cancer cells in separate experiments performed in triplicate. Magnification, ×200. **b** Number of invaded cells per field. Shown are the averages of invaded cells from 3 independent assays, with each assay cells in 5 random fields with ×200 magnification were counted. The numbers of cells transmitted through the membrane and standard deviations (SD) for BGC-823, SGC-7901, HGC-27, and MGC-803 are 6.1 ± 1.7, 13.2 ± 3.8, 32.8 ± 8.2, and 33.0 ± 8.2, respectively. **c** Comparison of the expression levels of molecular markers in four gastric cell lines

epithelial markers among these cells. We found that HGC-27 cells expressed relatively high level of the mesenchymal markers, vimentin, β-integrin and fibronectin; and a corresponding low level of epithelial marker, E-cadherin (Fig. 1c), the most pivotal effector molecule during EMT [12]. In MGC-803 cells, there were similarly high levels of fibronectin and vimentin, and low level of E-cadherin; but no detectable level of β-integrin was seen (Fig. 1c). Perhaps paradoxal, both MGC-803 and HGC-27 cells expressed fairly high level of β-catenin, another epithelial marker. It is possible that that heterogeneity derived from different progenitor cancer cells may differ at the level of β-catenin in various malignancies. These results indicated that the strikingly high invasion ability exhibited by the MGC-803 and HGC-27 cells was in well agreement with the acquirement of mesenchymal markers and the loss of epithelial marker in these cells.

TWIST overexpression promotes invasion and anti-apoptosis ability

To test whether TWIST expression is a key regulatory factor for cell migration, BGC-823 cells were transfected with pcDNA3.1-TWIST, which allows high expression of TWIST in mammalian cells. Forty-eight hours after transfection, TWIST level increased remarkably, compared to cells transfected with pcDNA3.1 empty vector (Fig. 2a). To test the effect of TWIST overexpression on BGC-823 cells'

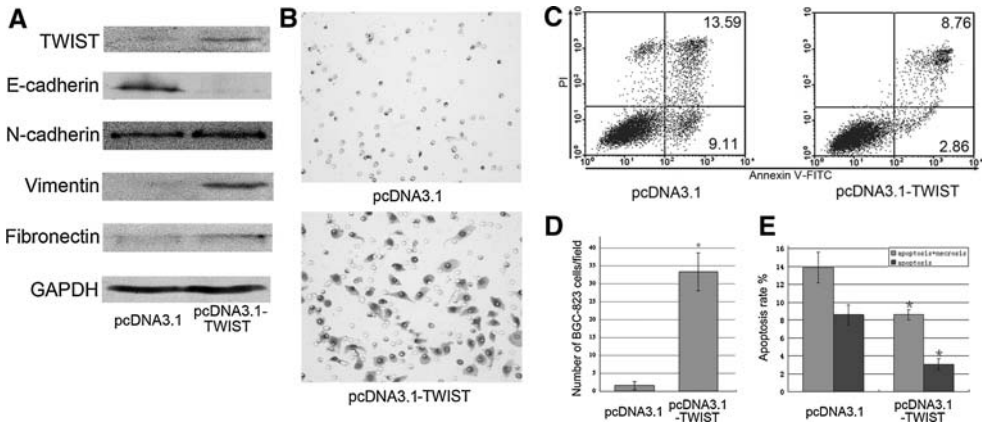

Fig. 2 TWIST overexpression in BGC-823 cells increased invasion and anti-apoptosis ability. **a** TWIST overexpression changes the expression of mesenchymal and epithelial markers. Forty-eight hours after transfection with pcDNA3.1-TWIST, cell lysates were blotted with indicated antibodies. **b**. Overexpression of TWIST increases invasion. After 16 h incubation, the invaded cells were fixed, stained and photographed at ×20 magnification. Invasion assays for untreated cells and for cells treated with vector or pcDNA3.1-TWIST, were carried out in parallel. Invasion ability for untreated cells is not different from cells treated with pcDNA3.1 vector and not shown. Representative figures are showed in three independent experiments. **c** Representative charts of FCM detecting apoptosis when TWIST was overexpressed. In the scatterplots, scatter in upper right quadrant represents the percentage of Annexin V and PI dual positive cells, which were dead cells; scatter in lower right quadrant represents the percentage of Annexin V positive but PI negative cells, which are early apoptotic cells. **d** Number of invaded cells per field. Shown are the averages of invaded cells from 3 independent assays, with each assay we counted cells in 5 fields with ×200 magnification. The numbers of cells and SD for pcDNA3.1 and pcDNA3.1-TWIST transfected cells are 1.6 ± 1.1 and 33 ± 5.3, respectively. **e** TWIST overexpression increases resistance to arsenic oxide. Shown are the percentages of dead cells (gray column) and apoptotic cells (dark column) in three independent experiments. The rates of apoptotic cells in pcDNA3.1 and pcDNA3.1-TWIST transfected cells were 8.6 ± 1.1 and 3.1 ± 0.62, respectively ($P < 0.05$), while the rates of dead cells were 14 ± 1.7 and 8.6 ± 0.59 ($P < 0.05$). *Error bars*, ±SD

behavior, we performed invasion assay for pcDNA3.1-TWIST-transfected cells. As expected, TWIST overexpression strikingly improved the invasion ability of BGC-823 cells (Fig. 2b, $P < 0.0001$) to a level similar to that of MGC-803 and HGC-27 cells that sustain high level of TWIST (Fig. 2d). Consistent with the increased invasion ability, TWIST overexpressed cells showed an increase in mesenchymal markers such as vimentin and fibronectin; but a decrease in epithelial marker, E-cadherin (Fig. 2a). These results indicate that the migration rate is responsive to and the cell surface markers are susceptible to the change of cellular TWIST level in BGC-823 cells.

In order to determine the protection activity of TWIST on apoptosis, 48 h after transfection with pcDNA3.1-TWIST or control vector, BGC-823 cells were treated with arsenic oxide, an effective apoptosis inducer, for 6 h to induce apoptosis. Apoptosis was measured by flow cytometry (FCM) after Annexin-V staining, which measures the translocation of phosphatidylserine from the inner to outer layer of plasma membrane, one of the earliest detectable events in the induction of apoptosis [24]. BGC-823 cells transfected with pcDNA3.1 control vector displayed fairly high percentages for both dead cells (upper-right quadrant) and early apoptotic cells (lower-right quadrant) (Fig. 2c). In contrast, BGC-823 cells transfected with pcDNA3.1-TWIST showed higher level of resistance to arsenic oxide, as shown by significantly decreased percentages of dead and early apoptotic cells ($P < 0.05$ respectively, Fig. 2e). These results indicate that high level of TWIST efficiently increased cells' resistance to apoptosis, and possibly necrosis, induced by chemotherapeutic drugs such as arsenic oxide in gastric cancer cells in vitro.

TWIST depletion causes depressed migration activity, decreased invasion ability and increased apoptosis sensitivity

Overexpression of TWIST in BGC-823 cells was found to reduce cells' apoptotic and necrosis rate upon response to arsenic oxide treatment. To learn more about the correlation of TWIST level and drug-resistance, we knocked down TWIST and monitored the rates of cell death responding to drug treatment in MGC-803 and HGC-27 cells, which express high level of TWIST. To do that, we transfected MGC-803 and HGC-27 cells with plasmids ShTWIST1, ShTWIST2, or ShScramble; and cell colonies that had stably integrated substrate containing a shRNA expression cassette, which can either generate siRNA specifically targeting *TWIST* gene (siTWIST1 and siTWIST2) or not (siScramble). Expression of ShTWIST1 or ShTWIST2 reduced TWIST level more than 80%, while expression of ShScramble did not affect TWIST level, in both MGC-803 (Fig. 3a) and HGC-27 cells (Fig. 3b). The morphological features of these cells were characterized

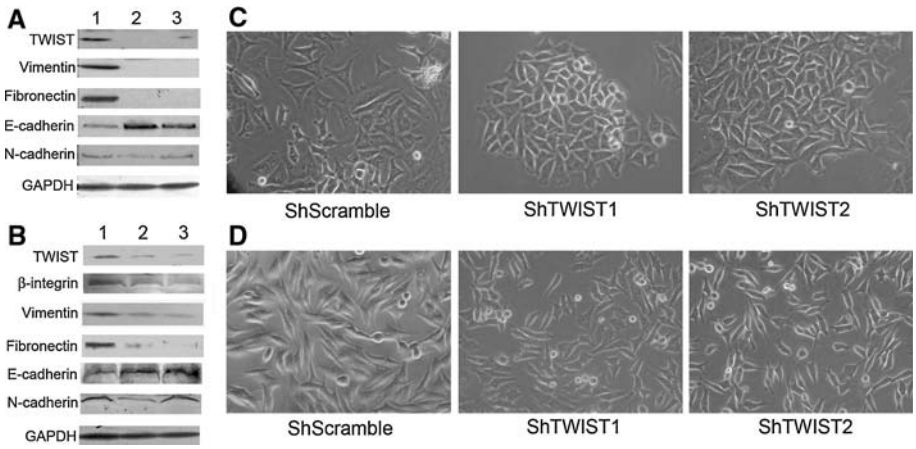

Fig. 3 TWIST knockdown leads gastric cancer cell to MET-like morphologic and molecular changes. **a** and **b**, The expression of epithelial and mesenchymal markers in MGC-803 (**a**) and HGC-27 (**b**) cells after TWIST depletion by ShTWIST1 or ShTWIST2. The changes of mesenchymal and epithelial markers were detected by Western-blot with indicated antibodies. Lane 1, ShScrmable. Lane 2, ShTWIST 1 and lane 3, ShTWIST 2. **c** and **d**, MET like phenotype of MGC-803 (**c**) and HGC-27 (**d**) cells after TWIST knockdown by ShTWIST1 or ShTWIST2. Pictures were taken when the cells reached exponential phase of growth

using photographing. As shown in Fig. 3c, inactivation of TWIST in MGC-803 cells exhibited dramatically morphologic changes from scattered and fibroblast-like shapes to tightly packed cobblestone, flat cell body and smaller cell volume morphology. Similar phenomenon was observed in HGC-27 cells after TWIST depletion (Fig. 3d). In addition, up-regulation of epithelial markers such as E-cadherin and down-regulation of mesenchymal markers such as vimentin and fibronectin were also found in TWIST depleted MGC-803 cells (Fig. 3a). Hence, both morphology and molecular changes indicate that TWIST depletion resulted in mesenchyme-to-epithelium transition, a reverse phenotype of EMT.

Then we asked whether TWIST depletion in MGC-803 cells can affect their migration rate. As shown in Fig. 4a, MGC-803 cells transfected with ShScramble efficiently migrated into the wound. In contrast, cells transfected with ShTWIST1 or ShTWIST2 showed markedly reduced migrating ability. Twenty-four hours after wounding, ShScramble-transfected cells had nearly healed the wound, but not ShTWIST1 or ShTWIST2-transfected cells. Knockdown of TWIST with either ShTWIST1 or ShTWIST2 leads to significant reduction of wounding rate in MGC-803 cells ($P < 0.05$, Fig. 4d). Once again, these results indicated a positive correlation between TWIST level and migration rate in gastric cancer cells. To test whether TWIST is related with EMT by controlling the gastric cancer cell transition through regulating EMT, BD Matrigel chamber invasion assay was performed and the results were shown in Fig. 4b and e. After incubation, many cells transfected with ShScramble transited through the Matrigel and grew on the lower side of the membrane, compared with much fewer cells transfected with ShTWIST1 or ShTWIST2 ($P < 0.01$). Thus, the invasive potential of MGC-803 cells was substantially reduced by TWIST depletion. The changes of these phenotypes after TWIST depletion occurred in another gastric cancer cell line HGC-27, which also expresses high level of TWIST (supplementary document 1). The reduction of the transition rate and the resident density in TWIST-depleted cells strongly suggests that TWIST is a pivotal regulator of metastasis process in gastric cancer.

To test the correlation of apoptosis and TWIST level in MGC-803 and HGC-27 cells, apoptosis was induced by treatment with arsenic oxide and assessed by Annexin-V staining as shown above. In MGC-803 cells transfected with ShScramble, the percentage of dead cells was significantly lower than that in cells transfected with ShTWIST1 or ShTWIST2 ($P < 0.01$ respectively, Fig. 4c, f). However, the percentage of early apoptotic cells after ShScramble treatment was marginally but significantly higher than ShTWIST1 or ShTWIST2 treatment (Fig. 4c, f). Similar to MGC-803 cells, ShTWIST1 or ShTWIST2-mediated TWIST depletion caused an increase of dead cells in HGC-27 cells compared with ShScramble treatment ($P < 0.01$). In addition, the rate of apoptotic ShScramble-treated cells was modestly lower than ShTWIST1 or ShTWIST2-treated cells. The variable percentages of apoptotic cells in MGC-803 and HGC-27 cell suggested that different gastric cancer cells show distinct sensitivity to the programmed cell death induced by arsenic oxide. The consistency for the direct correlation between apoptosis rate and TWIST level in TWIST-overexpressed BGC-823 cells and TWIST-depleted MGC-803 or HGC-27 cells indicate that TWIST is a key modulator of apoptosis in gastric cancer cells.

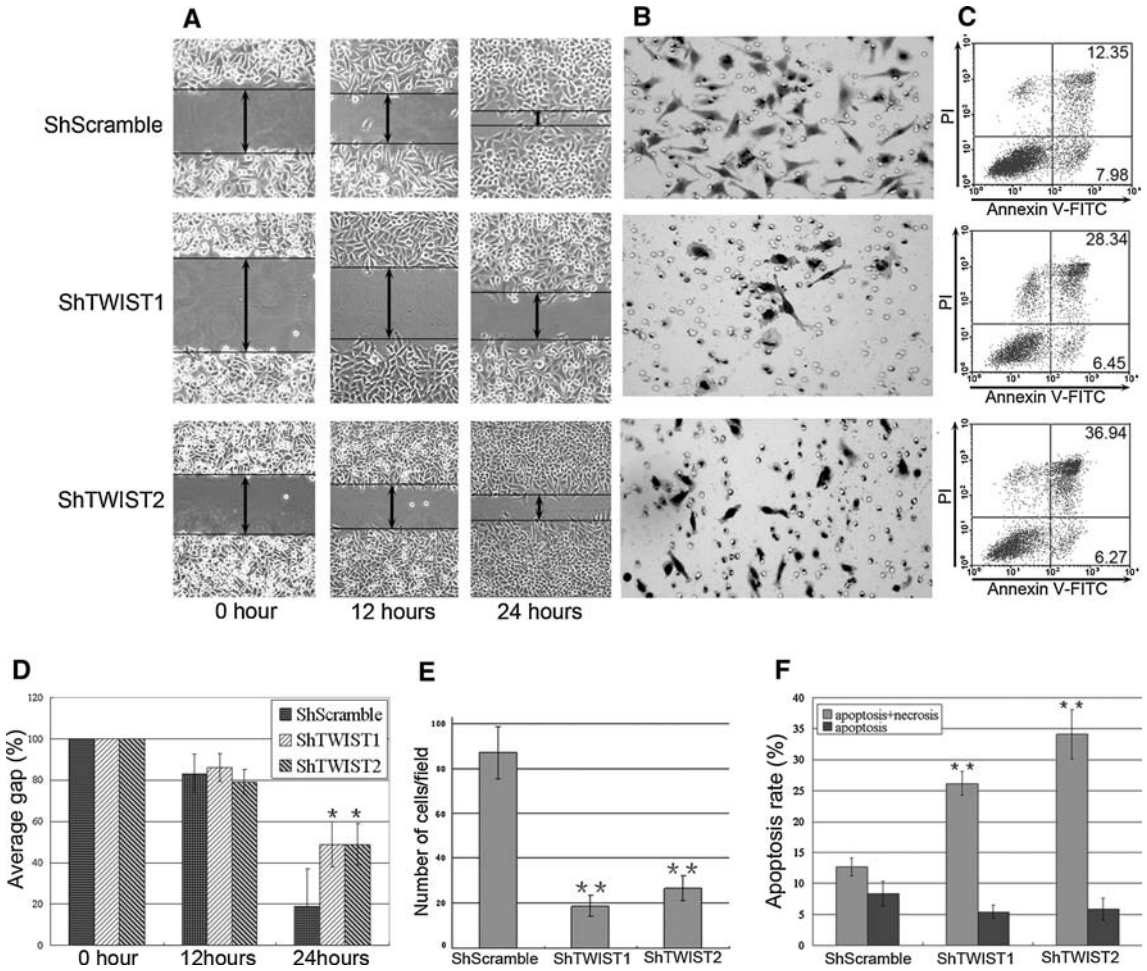

Fig. 4 TWIST knockdown results in decreased invasion and anti-apoptosis ability in MGC-803 cells. **a** Wound healing assay showed reduced migrating ability of TWIST depleted cells. Cell monolayers were selectively cultured in 6-well plate till 100% confluence. Photos of the same wound location were taken at different incubating duration as indicated. **b** Representative photograph of transwell assays showed reduced invasion ability brought by TWIST depletion. Invasion assays for untreated cells and for cells treated with ShScramble, ShTWIST1, or ShTWIST2, were carried out in parallel. Invasion ability for untreated cells is not different from cells treated with ShScramble and not shown. **c** Decreased apoptosis resistant ability of TWIST depleted cells. The procedure for apoptosis assay was described in Fig. 2C. Representative FCM diagrams from three independent experiments were showed. **d** Relative width of the wounds. The original wound width is arbitrarily defined as 100%. The widths in cells treated with ShScramble, ShTWIST1, or ShTWIST2 were 83 ± 9.3, 86 ± 6.7, and 79 ± 6.1, respectively after 12 h; and 19 ± 18, 49 ± 11, and 49 ± 10, respectively ($P < 0.05$, compared with ShScramble) after 24 h. Data shown are the averages of three measurements. **e** Number of cells moved through the membrane. Cells were counted 24 h after wound assay. The number of cells in ShScramble-treated is 87 ± 12, but they are 19 ± 4.8 and 27 ± 5.5 ($P < 0.01$) in ShTWIST1- and ShTWIST2-treated, respectively. **f** Changes of dead and apoptotic cells after TWIST knockdown. The rates of dead cells in total amount of ShScramble, ShTWIST1 and ShTWIST2 cells was 12.69 ± 1.45, 26.15 ± 1.94 and 34.09 ± 3.97, respectively (* $P < 0.05$ compared with ShScramble). And the ratio of each early apoptotic cells was 8.39 ± 2, 5.46 ± 1.02 and 5.88 ± 1.75 (no statistical significance found when compared each ShTWIST with ShScramble). *Error bars*, ±SD

TWIST knockdown results in cell cycle arrest

It has been reported that silencing of *TWIST* expression induces G1 phase cell cycle arrest of several types of cancer cells [25] through regulating the activity of Cyclin D [26]. To address whether cell cycle is regulated by TWIST in gastric cancer cells, we analyzed the cell cycle distribution of MGC-803 and HGC-27 cells before and after TWIST depletion. In MGC-803 cells, TWIST knockdown leads to remarkable increase of G1 phase cells ($P < 0.01$, Fig. 5a, b; Supplementary document 2). In TWIST-depleted HGC-27 cells, however, a striking percent of cells were arrested at G2 phase rather than G1 phase ($P < 0.01$, Fig. 5c, d). These results suggest that TWIST contributes to the cell cycle arrest in gastric cancer cells, while the stage of cells arrested could differ among various types of cells.

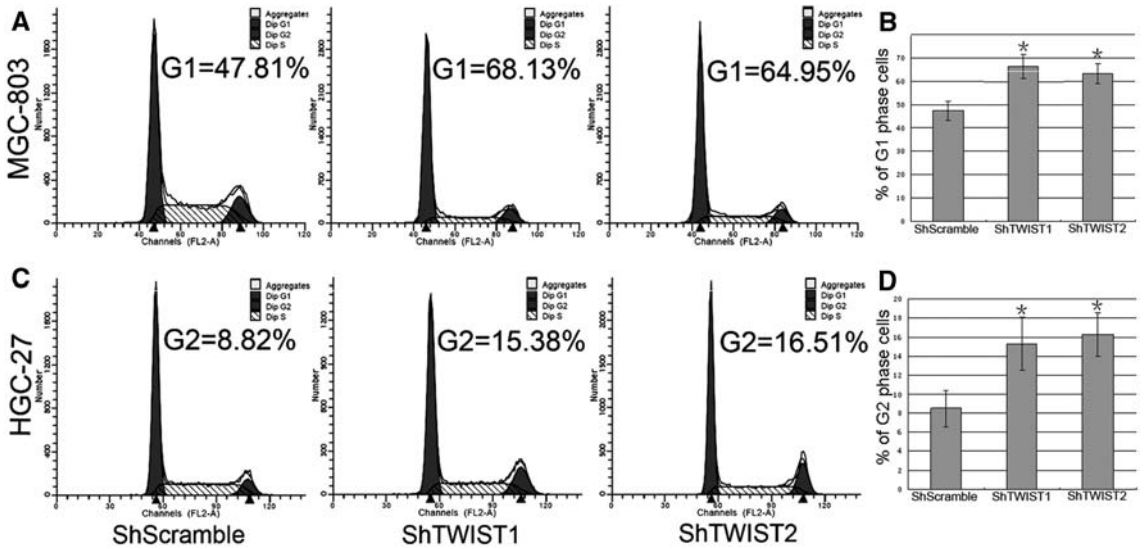

Fig. 5 TWIST knockdown induces cell cycle arrest. **a** TWSIT depletion induced G1 stage arrest in MGC-803 cells. Shown are the representative FCM diagrams from three independent experiments. **b** The proportion of G1 phase cells in ShScramble, ShTWIST1 and ShTWIST2 cells was 47 ± 4.0, 66 ± 4.9 and 63 ± 4.3, respectively ($P < 0.05$ compared each ShTWIST with ShScramble). **c** TWSIT depletion induced G2 stage arrest in HGC-27 cells. **d** The proportion of G2 phase cells in ShScramble, ShTWIST1 and ShTWIST2 cells was 8.5 ± 1.9, 15 ± 2.8 and 16 ± 2.3, respectively ($P < 0.05$ compared each ShTWIST with ShScramble). *Error bars*, ±SD

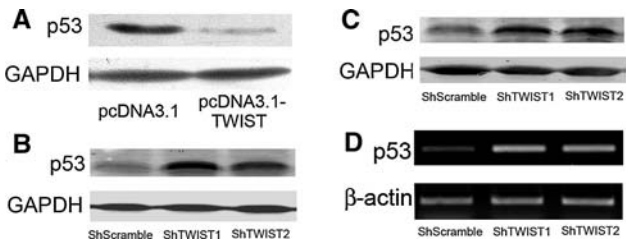

Fig. 6 TWIST regulates cellular p53 level. **a** Overexpression of TWIST in BGC-823 cells decreases p53 level. **b** and **c** TWIST depletion increases p53 level in MGC-803 (**b**) and HGC-27 (**c**) cells. **d** RT–PCR products of p53 mRNA in HGC-27 cells. RT–PCR was performed using the total RNA isolated from the ShScramble, ShTWIST1, or ShTWIST2 treated cells

TWIST regulates p53 expression

Tumor suppressor p53, encoded by *TP53* gene, plays a critical role on preventing tumor development. Its importance can be reflected by the high frequency (about 50%) of p53 inactivation due to mutations or deletion, in various types of cancers. In addition, p53 is involved in other pathways, including cell cycle arrest and response to genotoxic stress. We found that p53 is susceptible to the change of TWIST level in gastric cancer cells. For example, the p53 level was reduced to more than 3 fold by TWIST overexpression in BGC-823 cells (Fig. 6a), but elevated by over 100% in TWIST-depleted MGC-803 and HGC-27 (Fig. 6b, c). To further test whether the change of p53 is due to transcription regulation or protein stability, we measured the p53 mRNA levels. In HGC-27 cells, TWIST-depletion caused the induction of p53 mRNA by a few folds (Fig. 6d), suggesting that *TP53* gene expression is negatively regulated by TWIST.

Discussion

In this study, we characterized the TWIST level in four gastric cancer cell lines, in which MGC-803 and HGC-27 cells process relatively high level of TWIST, while it is low in BGC-823 and SGC-7901 cells. In breast cancer cell lines MCF-7 and MB-453 that express high level of TWIST, knockdown of TWIST leads to increased level of E-cadherin, a typical epithelial marker [5]. The close association of TWIST with highly invasive malignance has raised the question that whether TWIST could contribute to the invasive character of gastric cancer cells. In the highly invasive cell lines MGC-803 and HGC-27, knockdown of TWIST expression results in decreased migration content as well as morphological and molecular changes, characteristics of reversal of EMT. EMT has recently been verified as a key step in cancer metastasis and progression [11]. Meanwhile, overexpression of TWIST in BGC-823 cells leads to the highly invasive phenotype and up-regulation of EMT-linked markers. It has been reported that TWIST regulates cell motility and invasion of gastric cancer cell lines MKN45 and MKN-28 [27] through down-regulating E-cadherin and up-regulating N-cadherin and fibronectin production [28]. Collectively, the effects of—

overexpression in BGC-823 cells, and depletion of TWIST in MGC-803 and HGC-27 cells—on cells' invasion demonstrated that TWIST is a pivotal regulator of EMT and suggested that TWIST could play a key role in gastric cancer metastasis. Correlation of TWIST levels and EMT has been reported in other types of cancer cells [5, 29–34]. In addition, it is interesting that the level of N-cadherin changed little regardless the level of TWIST in above three cell lines, suggesting the N-cadherin is not a main downstream effector of TWIST signal pathway in gastric cancer, which is different from previous study [28].

Tumor growth is typically affected by two events: the rates of cell proliferation and cell death. Because cell growth arrest may provide cells the precondition for undergoing mesenchymal–epithelial transition [35], we investigated the changes of cell cycle distribution under different levels of TWIST. TWIST depletion leads to the G1 phase arrest in MGC-803 cells and G2 phase arrest in HGC-27 cells, suggesting that high level of TWIST is able to drive cell cycle progression. This is consistent with that TWIST overexpression results in repression of CHK1 and CHK2—two key proteins involved in G1-S and G2-M phase checkpoint—in prostate epithelial cells [36]. Furthermore, the rate of cell death responding to the treatment of chemodrug arsenic oxide was also associated with TWIST level. TWIST overexpression in BGC-823 cells leads to reduced rate of both apoptosis and necrosis. Consistent with that, TWIST depletion increases the rate of apoptosis and necrosis in MGC-803 and HGC-27 cells. In summary, we have identified a direct correlation between TWIST level and cell cycle arrest, apoptosis in gastric cancer cells.

At this moment, the mechanism of how TWIST affects cell cycle progression and apoptosis is un-identified yet. One possibility is that p53 is regulated by TWIST in these cells. Due to the multiple roles of p53 on cell cycle checkpoint, DNA damage response, regulation of gene expression, and apoptosis [37], the cellular level of p53 is strictly controlled and finely tuned to guarantee the normal functions of cellular pathways. The changes of p53 level in cells after TWIST overexpression or depletion were monitored in gastric cells. Typically, high level of TWIST—such as in MGC-803 and HGC-27 cells or TWIST-overexpressed BGC-832 cells—leads to reduced p53 level, while low level of TWIST causes accumulation of p53. The p53 activity is regulated by a complicated network, including regulation of transcription, post-translational modification, interaction between p53 and other proteins, and protein stability. In HGC-27 cells, TWIST-depletion increased p53 level as well as its mRNA level, suggesting that the increased p53 level is primarily due to the enhanced expression of *TP53* gene. These results also suggest that TWIST suppresses cell cycle arrest and apoptosis via negatively regulating p53. Furthermore, TWIST can also place its effect on regulating p53 via repressing the expression of $p19^{ARF}$ (referred to as ARF hereafter), as shown in mouse embryonic fibroblast [14]. ARF plays a critical role on stabilizing p53 via interaction with MDM2, which has ubiquitin E3 ligase activity and is thought to be the primary ligase for p53 [38]. Binding of MDM2 to p53 can transfer ubiquitin to p53 at its C-terminal, targeting p53 for proteasome-dependent protein degradation. Thus, a simplified model—wrong or right—is that TWIST causes reduced level of ARF, which in turn allows more MDM2 to bind p53, consequently causing p53 degradation. Indeed, several reports have demonstrated that the down-regulation of *ARF* gene expression is determinative for the inactivation of p53 in response to TWIST expression [14, 36]. The anti-apopotic effect of p53 might be subject to the modulation of other proteins via protein–protein interaction directly or indirectly. For example, AKT2 was found to be a repressor of p53 [39] and positively correlated with TWIST in cells used in this study. AKT represses the function of p53 through phosphorylating and subsequent nuclear localization of MDM2, which in turn binds with p53 and increases its nuclear export, leading to a decrease of cellular p53 level by ubiquitinated degradation [40]. Indeed, AKT2 protein is strikingly increased in TWIST overexpression BGC-823 cells and decreased after TWIST had been specifically depleted in both MGC-803 and HGC-27 cells (our unpublished data). As *AKT2* gene expression is activated by TWIST via its binding to the E-box of *AKT2* promoter [5], cellular AKT2 is dependent on the level of TWIST. Thus, in gastric cancer, the rates of apoptosis and cell cycle progression in cells with high level of TWIST are, in principle, modulated by the elevated AKT2 level and subsequently decreased level of p53. Collectively, TWIST likely regulates p53 activity in gastric cancer cells through multiple pathways, including transcriptional suppression of *TP53* gene expression, repression of the p53-stability positive modulator ARF, and induction of p53-stability negative modulator AKT2, among other possibilities.

In summary, it is evident that TWIST—in a few lines of gastric cancer cells—can affect the rate of migration, cell cycle progression, apoptosis, and pattern of gene expression. We also found that p53 level is sensitive to TWIST, providing a testable model that TWIST affects tumorigenesis of gastric cancers via regulating the tumor suppressor, p53. What we do not know yet is that whether TWIST level is directly correlated with the malignancy of gastric cancers in vivo. Future studies especially those in mouse models are warranted to address this question and to establish the molecular basis for the tumorigenesis of gastric cancers.

Materials and methods

Plasmid constructions

Plasmid pcDNA3.1-TWIST was generated by cloning *TWIST* cDNA into eucaryotic expression vector pcDNA3.1 vector. *TWIST* cDNA was synthesized using the following primers: TWISTF: 5′-CTAGCTAGCGAGAGATGATGC AGGACGTGTC-3′; TWISTR: 5′-CGGAATTCCTAGTG GGACGCGGACATG-3′. The *Nhe I* and *EcoR I* sites added to the primers were used for cloning *TWIST* cDNA. For TWIST silencing, pSUPER RNAi system (Oligoengine) was used to construct *TWIST* RNAi vectors. Two pairs of oligo inserts are designed with the sense sequences shown as follows: 5′-GATCCCCAAGCTGAGCAAGAT TCAGACCTTCAAGAGAGGTCTGAATCTTGCTCAGC TTTTTTTA-3′ and 5′-GATCCCCAGGTACATCGAC TTCCTCTACTTCAAGAGAGTAGAGGAAGTCGATGT ACCTTTTTTA-3′. The underlined sequences are identical to the *TWIST* gene, and are anticipated to be processed to siRNAs specifically targeting *TWIST* gene in cells. These pairs of oligos were cloned into mammalian expression vector pSUPER Retro Puro at the unique *Bgl II* and *Hind III* sites, generating ShTWIST1 and ShTWIST2 respectively. The ShScramble vector, whose insert does not match any known human cDNA, was used as control. All of the recombinant plasmids used in this study were verified by DNA sequencing.

Cell culture and transfection

All cells were cultured in RPMI1640 medium with 10% FBS (Gibco) and in 5% CO_2 with a humidified atmosphere at 37°C. Lipofectamine 2000 (Invitrogen) was used for transfection. Briefly, 30 μl of transfection reagent was mixed with 12 μg of pcDNA3.1-TWIST vector or control vector respectively; and the mixture was added into 8 ml RPMI1640 medium per 10 cm culture dish, at over 90% of cell confluence. For TWIST RNAi expression, 4 μg of ShTWIST1, ShTWIST2, or ShScramble was mixed with 10 μl of transfection reagent; the mixture was added into 2 ml RPMI1640 medium per well of 6-well plate at about 70% cell confluence. Six hours later, cells were refed with fresh medium and continued for culture for another 24 h. Then the cells were cultured with medium containing puromycin at the final concentration of 0.5 μg/ml for HGC-27 and 0.3 μg/ml for MGC-803, respectively. After 5–6 days' selection, cell colonies were randomly picked up and transferred to new dishes for continuous culture in selective medium. The cultures were fed with fresh selective medium once every 2 days. When the cell reached exponential growth, WB was performed to identify the expression level of TWIST. Two cell colonies whose TWIST expression were most intensively repressed with different target RNAi fragment were assigned as ShTWSIT1 and ShTWIST2 respectively and subjected to further experiments.

Cell invasion assay

Invasion assays were done in BD precoated (Figs. 1, 2) or freshly coated (other Figures) BioCoat Matrigel Invasion chambers with polyethylene terephthalate filter inserts for 24-well plates containing 8 μm pores with a thin layer of MATRIGEL basement membrane matrix (BD Bioscience). After 16 h starvation, 5×10^4 cells were plated in 100 μl of 0.5% bovine serum albumin (BSA)-RPMI1640 into the upper chamber. The lower chamber was filled with 500 μl of 10% FBS-RPMI1640. After 16 h incubation, noninvaded cells in the inserts were removed with cotton swabs. The invaded cells on the underside of membrane were treated with fixative solution (100% methanol) for 1 min and stained for visualization by hematoxylin (2 min) and eosin (40 s) sequentially. Then the invaded cells were photographed and counted in 5 random microscopic fields with 20× objective per well. For each cell line, 3 duplicate invasion wells were used to confirm the identity. Experiments were performed in triplicate.

Cell cycle analysis

One million cells were seeded in 6-well plate and cultured in serum free RPMI1640 medium for cell synchronization. After starving for 16 h, cells were washed with PBS and incubated in fresh 10% FBS-RPMI1640 medium at 37°C for another 24 h. At the appropriate time interval, after being washed in ice cold PBS twice, cells were fixed with ice cold 70% ethanol for 30 min, then resuspended in 500 μl solution containing 50 μg/ml propidium iodide (PI, BD Bioscience) and 50 μg/ml RNaseA for 30 min at 37°C. Flow cytometry (FCM) analyses were performed with FACSCalibur™ using standard CELLQuest™ acquisition/analysis software (BD Bioscience). After gating out cell debris and fixation artifacts, FCM analysis allowed for the discrimination of DNA contents. G0/G1, S and G2/M populations were quantified using the ModFiT™ program (BD Bioscience).

Wound healing assay

5×10^5 cells were seeded into a 6-well plate and fed with fresh selective medium containing 0.5 μg/ml of puromycin for HGC-27 and 0.3 μg/ml for MGC-803, respectively. Cell monolayers were wounded with a 10 μl plastic tip. The migration was followed for 12 h, 24 h respectively and photographed using a NiKon camera mounted on a

microscope (Olympus) with a 10× modulation objective. Experiments were performed thrice.

Apoptosis induction

5×10^5 of cells were seeded in 6-well plate. After 24 h, arsenic oxide (Sigma–Aldrich) was added to achieve the final concentrations of 3 μM for HGC-27 and MGC-803, and 2 μM for BGC-823. After 6 h incubation periods, the supernatant was collected, and detached cells were spun down. Adherent cells were collected after trypsin treatment. Adherent and detached cells were pooled, washed with PBS containing 1% BSA, and subjected to the respective apoptosis detecting assays. For each assay, 2×10^4 cells were applied.

Apoptosis detection

The assay for apoptosis detection was performed using Annexin V-FITC kit (Sigma–Aldrich) according to the instruction of the manufacturer. Briefly, cells were washed twice with PBS and resuspended in 1× Binding Buffer at a concentration of 1×10^6 cells/ml. Five microliter of annexin V FITC Conjugate and 10 μl of PI solution were added sequentially to each 500 μl of cell suspension, and the mixture was incubated in dark at room temperature for 10 min. Fluorescent cells were measured in a FACSCalibur (BD Bioscience). The percentage of viable apoptotic cells was calculated from the annexin V single positive scatterplot. Non-viable apoptotic and necrotic cells were determined by both the PI Solution and annexin V positive scatterplot.

Western blotting

Cells were washed for three times with ice-cold PBS. Cell lysates were prepared with RIPA buffer containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS 50 mM Tris–Cl, pH 7.4, 20% (V/V) cocktail protease inhibitors (Sigma–Aldrich). After a brief sonication on ice, cell lysate was incubated on ice for 1 h, and centrifuged at 13,000*g* for 30 min at 4°C to remove insoluble debris. Whole cell lysates were treated by boiling in loading buffer containing and resolved by SDS–PAGE, then electrophoretically transferred to membranes (Immobilon-P, Millipore). Membranes were pre-blotted over night in TBS-T buffer (3 g/l Tris-base, 8 g/l NaCl, 0.2 g/l KCl, 0.1%Tween-20, pH 7.4) containing 5% skim milk. After that, the membranes were incubated with specific primary and second antibodies in TBS-T plus 5% skim milk for 1 h respectively at room temperature; and developed using enhanced chemical luminescence (ECL). The antibodies were purchased from Santa Cruz Biotechnology, Zhongshan Beijing Biotechnology, or Shanghai Kangchen Bio-tech.

Analysis of p53 mRNA level

Total RNA was isolated from the gastric cancer cells, and 1 μg of RNA was reversely transcribed into cDNA. A portion of the cDNA was quantitatively analyzed by PCR with *p53* specific primers. The primers are: 5′-GTGCAG CTGTGGGTTGATTC-3′ and 5′-CAGTGCTCGCTTAGT GCTCC-3′. Conditions for PCR were 94°C for 3 min, followed by 27 cycles of 30 s at 94°C, 30 s at 59.5°C and 40 s at 72°C, and an additional 5 min extension at 72°C at the end of cycles. The PCR product is a 498 bp fragment. Twenty microliter of the PCR product was resolved in a 1.0% agarose gel. The bands were scanned and the band intensities were assessed by using the QuantiScan software. Transcription levels of p53 were normalized to that of β-actin transcript which was amplified with the following primers: ACTCTTCCAGCCTTCCTTCC and CATACTCCTGC TTGCTGATCC.

Statistical analysis

Data were expressed as median and range and compared between groups using the F test to determine equal variances. Categorical variables were compared using student two tailed t test. $P < 0.05$ was considered statistically significant. All statistical analyses were done using statistics software (SPSS 11.0 for Windows; SPSS, Inc.).

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