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Original Article

ASPP2 suppresses invasion and TGF-β1-induced epithelial–mesenchymal transition by inhibiting Smad7 degradation mediated by E3 ubiquitin ligase ITCH in gastric cancer

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ABSTRACT

ASPP2 regulates cell polarity and cell–cell adhesion by binding to, and co-localizing with PAR3 at tight junctions. Here we show a novel role of ASPP2 in suppressing gastric cancer (GC) invasiveness. Immunoprecipitation and immunofluorescence analyses showed that ASPP2 promoted the recruitment of PAR3 to cell–cell junctions in GC cells. Diminished expression of ASPP2 and loss of junctional PAR3 localization were significantly associated with diffuse-type histology, deeper invasion depth, positive peritoneal dissemination and worse prognosis in primary GC. ASPP2 suppressed migration and invasion of GC cells *in vitro* and peritoneal dissemination of GC cells *in vivo* in a mouse model. ASPP2 suppressed epithelial–mesenchymal transition (EMT) induced by TGF-β1-Smad2/3 signaling in GC cells through suppression of the degradation of Smad7, a negative regulator of TGF-β1-Smad2/3 signaling, by interacting with the E3 ubiquitin ligase ITCH. In conclusion, ASPP2 suppresses invasion, peritoneal dissemination and TGF-β1-induced EMT by inhibiting Smad7 degradation mediated by ITCH.

Introduction

Gastric cancer (GC) is currently the third leading cause of cancer deaths in the world [1,2]. The vast majority of GCs are adenocarcinomas, which are usually grouped into intestinal-type gastric cancer (IGC) and diffuse-type gastric cancer (DGC) according to the Lauren classification [3]. IGC is histologically characterized by gland-like tubular structures formed from well differentiated adenocarcinoma cells. On the other hand, DGC is composed of poorly differentiated cancer cells without formation of gland-like structures, and of thick stromal fibrosis with high levels of TGF-β1 [4,5]. DGC has a worse prognosis than IGC, because peritoneal dissemination and lymph node metastasis are frequently observed in DGC. DGC is more common in young patients, with female predominance, and the incidence of DGC has progressively increased over the last three decades [6]. Progress in understanding the molecular mechanisms of DGC has been limited, although recurrent somatic *CDH1* (encoding E-cadherin) and *RHOA* mutations have been identified in DGC [7].

ASPP2, encoded by *TP53BP2*, was originally identified as a tumor suppressor and an activator of the p53 family [8–10]. Expression levels of ASPP2 are downregulated in several human cancers including hepatocellular carcinomas [11], head and neck cancers [12], and diffuse B cell lymphomas [13]. In gastric carcinogenesis, the cytotoxin associated gene A protein of *Helicobacter pylori*, which is related to increased risk of GC, interacts with ASPP2, resulting in disruption of its anti-apoptotic function in noncancerous gastric epithelia [14,15]. Recent emerging evidences indicate that ASPP2

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also acts as a key regulator of cell polarity. ASPP2 interacts with PAR3 at tight junctions, which are placed at the most apical side of the epithelial cell membrane in normal epithelial cells [16,17]. PAR3 forms the evolutionarily conserved partition-defective (PAR) complex, consisting of PAR3, PAR6, and aPKC, in epithelial cells [18–21]. ASPP2 regulates apico-basal polarity by recruiting the PAR complex at tight junctions through interaction with PAR3 in epithelial cells [16,17].

Epithelial mesenchymal transition (EMT) is a process by which epithelial cells acquire a mesenchymal cell phenotype. During the EMT process, cells gradually lose the epithelial characteristics of cell–cell adhesion and apico-basal polarity, and reorganize the cytoskeleton into a spindle-like cell shape, thereby gaining increased motility and invasiveness. Hence, EMT is considered to be a key process for tumor invasion [22,23]. The common molecular markers of EMT include loss of expression of epithelial markers including E-cadherin, and gain of expression of mesenchymal markers including vimentin. EMT can be induced by tumor microenvironment components such as TGF-β1 [24]. The transcriptional network for TGF-β1-induced EMT is mainly mediated by Smad2/3 signaling. Upon ligand binding, activated TGF-β receptors phosphorylate Smad2/3, which forms complexes with Smad4 that translocate from the cytoplasm into the nucleus [24,25]. Subsequently, the Smad complexes regulate the transcription of EMT associated genes. On the other hand, inhibitory Smad proteins such as Smad7 block TGF-β1 signal transduction. Smad7 inhibits Smad2/3 phosphorylation by forming a complex with the TGF-β receptor [26] or by mediating degradation of the TGF-β receptor [27].

We [28] and others [29–32] have shown that loss or mislocalization of PAR3, a binding partner of ASPP2, promotes metastasis in esophageal squamous cell carcinoma, breast cancer, and renal cell carcinoma. A recent study suggested that reduced ASPP2 expression facilitates EMT, tumor invasion and metastasis of breast cancer and hepatocellular carcinoma cells [33]. In the present

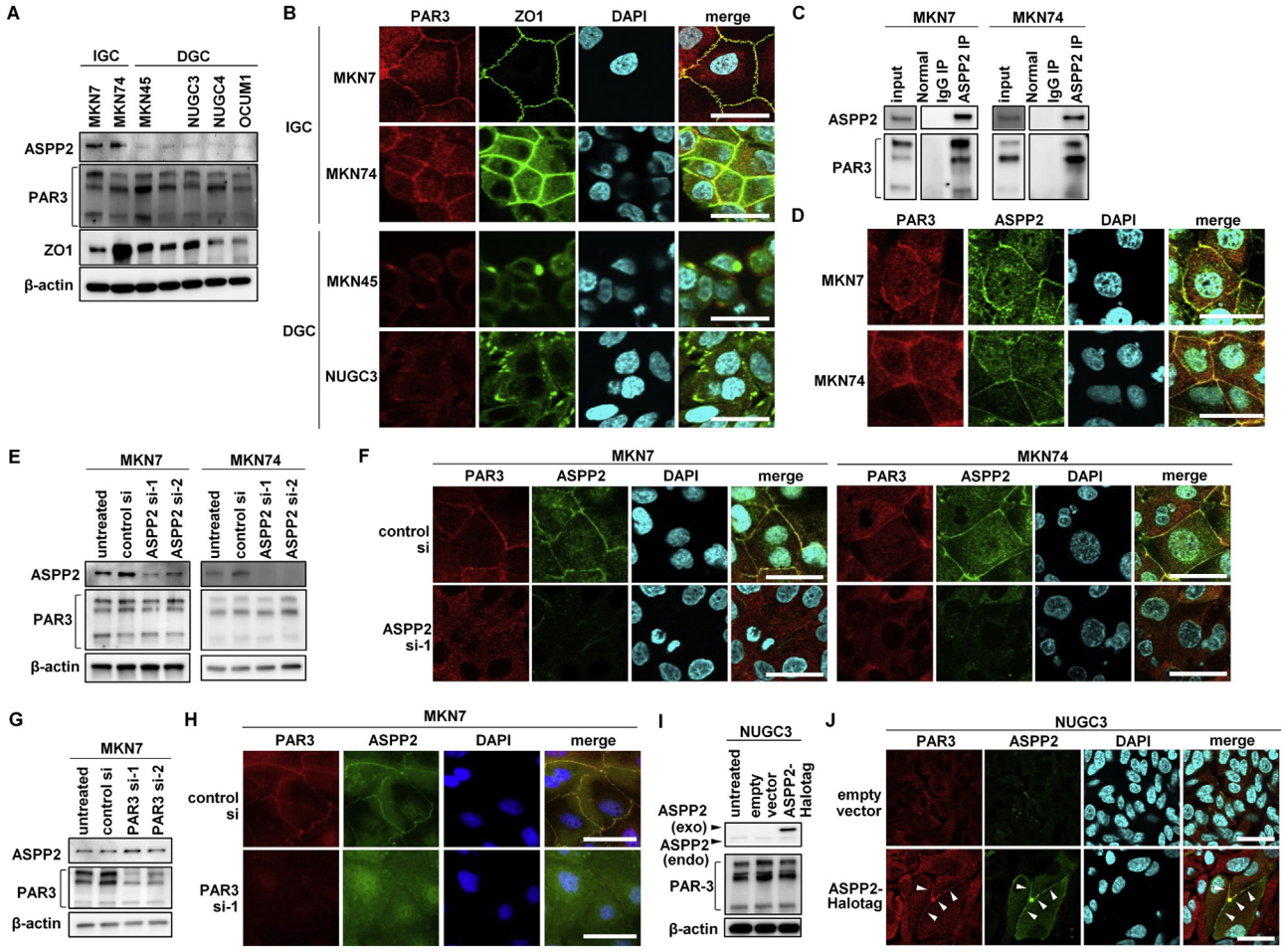

Fig. 1. ASPP2 controls junctional localization of PAR3 in GC cells. (A) Immunoblot analysis of ASPP2, PAR3, ZO-1, and β-actin, an internal control, in seven gastric cancer cells. Note that the immunoblot indicates three forms of the PAR3 protein with molecular weights of 180, 150, and 100 kDa. (B) Immunofluorescence analysis of MKN7, MKN74, MKN45 and NUGC3 cells that were triple-labeled with anti-PAR3 (red), anti-ZO-1 (green), and DAPI (blue; nuclei). (C) Examination of the interaction between ASPP2 and PAR3 in MKN7 and MKN74 cells by co-immunoprecipitation experiments. Endogenous ASPP2 was immunoprecipitated with an anti-ASPP2 rabbit polyclonal antibody, or normal rabbit IgG (negative control). ASPP2 and PAR3 in the precipitates were detected by immunoblotting. (D) Immunofluorescence analysis of MKN7 and MKN74 cells that were triple-labeled with anti-PAR3 (red), anti-ASPP2 (green), and DAPI (blue; nuclei). (E) Immunoblot analysis of the indicated proteins in MKN7 or MKN74 cells transfected with control siRNA or ASPP2 siRNA. (F) Immunofluorescence analysis of MKN7 or MKN74 cells treated with control siRNA or ASPP2 siRNA. Cells were labeled as in (D). (G) Immunoblot analysis of the indicated proteins in MKN7 cells transfected with control siRNA or PAR3 siRNA. (H) Immunofluorescence analysis of MKN7 cells treated with control siRNA or PAR3 siRNA. Cells were labeled as in (D). (I) Immunoblot analysis of the indicated proteins in NUGC3 cells transfected with an empty vector or an ASPP2-Halotag expressing vector. Exogenous (exo) and endogenous (endo) ASPP2 are indicated by arrowheads. (J) Immunofluorescence analysis of NUGC3 cells transfected with an empty vector or an ASPP2-Halotag expressing vector. Cells were labeled as in (D). Junctional localization of ASPP2 or PAR3 is marked with white arrowheads. All scale bars: 50 μm.