

PUMA Kills Stem Cells to Stall Cancer?

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Abstract

Apoptosis evasion is a hallmark of human cancer. PUMA is a BH3-only Bcl-2 family protein that mediates both p53-dependent and independent apoptosis. However, its role in tumor suppression had not been well established. Our recent work provides direct evidence that PUMA plays an important role in suppressing intestinal tumorigenesis in two mouse models including (i) the azoxymethane (AOM)/dextran sulfate sodium salt (DSS)-treated mice and (ii) *APC*^{Min/+} mice. The activities of PUMA appeared to be in the intestinal stem cells, and involve both p53-dependent response to DNA damage, and p53-independent mechanisms triggered by inflammation. Our data suggest that the interplay between different apoptotic pathways in intestinal stem cells underlie the initiation of intestinal carcinogenesis, and should be considered in the context of cancer prevention and therapy.

Keywords: PUMA; p53; Apoptosis; Colon cancer; Stem cells

Introduction

The multi-step carcinogenesis process involves the accumulation of a series of genetic and epigenetic alterations (1, 2). A number of features distinguish cancer cells from their normal counterparts. Resistance to apoptosis is a hallmark of virtually all types of human cancer, and leads to increased risk of clonal expansion of cells containing neoplastic alterations (3). Colon cancer is perhaps the most well studied model that illustrates this

multi-step nature (2). Apoptosis, a major mechanism regulating turnover of intestinal epithelial cells, is progressively inhibited in colon carcinogenesis (4, 5). This is in part attributed to frequent mutations in tumor suppressors *APC* (*adenomatous polyposis coli*) and *p53*, and in oncogenes *β-catenin*, *K-ras*, *BRAF*, and *PI3K* (2). In addition, overexpression of antiapoptotic proteins such as Bcl-2, Bcl-xL, and survivin are also common in cancer (6). These alterations can directly or indirectly suppress apoptosis and subsequently drive other tumorigenic events leading to cancer (3). However, direct *in vivo* evidence supporting the role of apoptosis in tumor suppression, especially in solid organs, is generally scarce. Using mice deficient in the proapoptotic Bcl-2 family protein PUMA, we demonstrated that both p53-dependent and independent apoptosis contribute to the suppression of malignancies in the intestinal tract (7).

Material and Methods

The procedures of animal work and various methods for Western blotting, caspase 3 and BrdU immunohistochemistry have been previously described (7). The HCT 116 and its isogenic *p53* knockout (*p53* KO) cells have been previously described (8).

Results and Discussion

The role of BH3-only proteins in tumor suppression

The Bcl-2 family proteins are evolutionarily conserved key regulators of apoptosis which include both antiapoptotic and proapoptotic members. PUMA (p53 upregulated modulator of apoptosis) was originally identified as a transcriptional target of p53 and a BH3-only protein that induces rapid and potent apoptosis in various cancer cells (9-12).

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Similar to other BH3-only proteins, PUMA serves as a proximal signaling molecule which transduces death signals via multidomain Bcl-2 family members to induce mitochondrial dysfunction and caspase activation (13, 14). PUMA primarily acts to indirectly activate Bax and/or Bak by relieving the inhibition of these proteins by antiapoptotic Bcl-2 family members (15). *PUMA*-deficient human or mouse cells from various tissue origins including the intestinal tract have profound apoptotic deficiencies in response to a wide variety of stresses, establishing its principle role in promoting apoptosis (13, 16-19).

The Bcl-2 family proteins are linked to cancer. Genetic alterations of Bcl-2, the founding member of the family, are responsible for a subset of leukemia. Several prosurvival family members, including Bcl-2, Bcl-xL and Mcl-1, are frequently overexpressed in colon cancer and other human malignancies. In rare cases, the proapoptotic member *BAX* is mutated in mismatch repair-deficient colon cancer (20). On the other hand, the BH3-only proteins including PUMA are not inactivated by mutations in cancer (21, 22). Several lines of evidence however suggest that the function of PUMA is frequently compromised in cancer as a result of p53 dysfunction (23), overexpression of the antiapoptotic Bcl-2 family members (24), reduced expression due to promoter methylation (25), or activated oncogenic kinase pathways including PI3K/AKT (26-28). Our work now provides direct evidence for a role of PUMA in suppressing carcinogenesis in two intestinal tumor models, the AOM/DSS carcinogenic model and the *APC/min* genetic model (7). Our findings are in agreement with previous studies suggesting a tumor suppressor role of PUMA (29, 30) and Bim (31), another BH3-only protein, in lymphomagenesis in mice. Together, these observations establish a critical role for BH3-only proteins in tumor suppression and their deregulation in cancer in the absence of genetic alteration.

Apoptosis induction as a critical tumor suppressor function of p53

The p53 tumor suppressor is essential for preventing inappropriate cell proliferation and for maintaining genome integrity following genotoxic stress (32, 33). The function of p53 is multifaceted and not restricted in the nucleus, while transcriptional activation by p53 appears to be essential for its proapoptotic function *in vitro* and *in vivo* (34). The great majority of tumor-derived p53 mutants are defective in apoptosis induction and

unable to transactivate proapoptotic downstream targets such as PUMA and Noxa (23). Genes encoding proteins involved in virtually every step of apoptosis can be regulated by p53 (23). However, a lingering question is how does p53-dependent apoptosis stop cancer? Loss of p53 in mice results in early onset lymphomas and animal death, which provides little insights on how it acts as a tumor suppressor in solid organs (35). There is little doubt that PUMA accounts for much of the proapoptotic activity of p53 in response to DNA damage *in vivo*, and the induction of PUMA by DNA damage is strictly p53-dependent (9, 11, 13, 36, 37). Nevertheless, *PUMA*-deficient mice did not develop spontaneous tumors more readily compared to their WT counterparts under pathogen free conditions (16, 17).

Our recent work indicates that the tumor suppressive activities of PUMA are more pronounced following DNA damage through a p53-dependent mechanism in the intestinal tract (7). The induction of PUMA by AOM in colonic epithelial cells in culture was also dependent on p53 (Figure. 1A). This would suggest that p53 guards against DNA damage to suppress oncogenesis through induction of PUMA and apoptosis. Loss of PUMA or p53 significantly blocked AOM-induced apoptosis while only minimally impacting cell proliferation, if at all (7) (Figure. 1B). Our findings are consistent with an earlier finding that an intact p53 pathway suppressed AOM-induced colon carcinogenesis (38). However, another study suggested that p53-dependent apoptosis in response to DNA damage is not important for tumor suppression (39). In this study, the authors used a mouse model in which p53 can be switched on or off to show that the p53-mediated pathological response to whole-body irradiation is not required for suppression of radiation-induced lymphoma. This conclusion perhaps does not apply to carcinomas. Moreover, Noxa, another BH3-only protein, can sometimes substitute or work together with PUMA to mediate p53-dependent apoptosis following DNA damage (17, 37). It would be of interest to explore whether Noxa is also involved in p53-dependent tumor suppression in similar models.

Does colon cancer arise from mutated colonic stem cells?

The intestinal epithelium is the fastest renewing tissue in the human body and is regenerated by stem cells located at the bottom of the crypts, which supply all differentiated cell types (40).

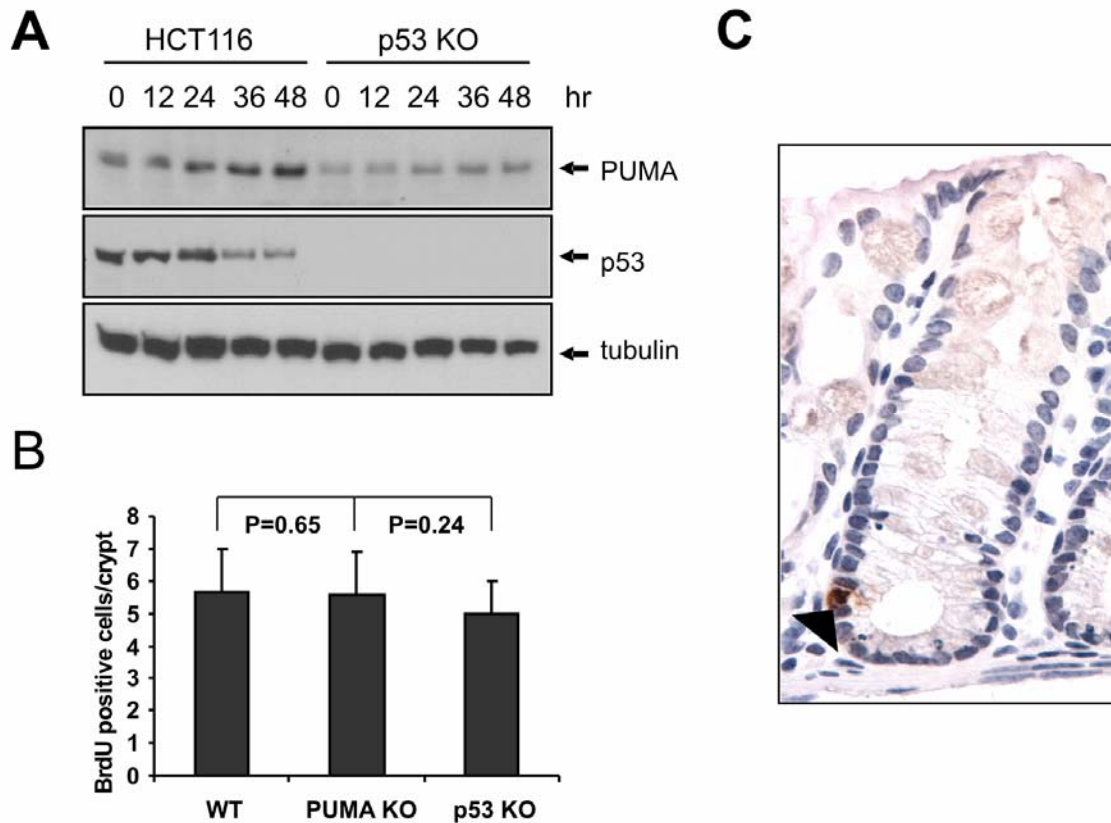


Figure 1. p53-dependent PUMA induction contributes to AOM-induced apoptosis but not proliferation. (A) The parental or p53 KO HCT116 cells were treated with 20 ng/ml AOM for the indicated time. The levels of PUMA, p53 and tubulin were analyzed by Western blotting. (B) The proliferation of the colonic crypts was determined by BrdU immunohistochemistry (IHC) in WT, PUMA KO and p53 KO mice 72 hr following AOM injection. (C) Active caspase-3 (brown, arrow head) in the colonic crypts 8 hr after AOM injection was detected by IHC.

Differentiated enterocytes are constantly removed by apoptosis after migrating to the top of the villus (4, 41). What is the cellular origin of intestinal tumors? Are they derived from stem cells that have endured critical mutations in a certain sequence (1), or from more differentiated cells that have acquired mutations conferring stem cell-like properties? Given the limited life span of differentiated cells in the intestinal tract, and the requirement of a number of critical events for tumorigenesis, most believe that such events must occur in the stem cells. However, direct evidence was only available recently upon the discovery of intestinal stem cell markers. Several elegant genetic studies indicate that inactivating *APC* in the intestinal stem cells, but not in rapidly proliferating progenitors, results in rapid intestinal tumorigenesis in mice (42, 43). In our study, these cells are undergoing PUMA- and p53-dependent apoptosis following AOM treatment

(Figure 1C) (7), suggesting that removal of damaged stem cells protects against cancer and such cells as the origin of colon cancer. Moreover, PUMA deficiency increases the incidence of the precursor lesion aberrant crypt foci (ACF) induced by AOM in the colon, or that of microadenomas in the small intestine of *APC^{Min/+}* mice, supporting a stem cell origin in tumor initiation. It remains to be clarified whether or how PUMA contributes to the removal of colonic stem cells under oncogenic stress, presumably upon the loss of the other allele of *APC*. Stem cell lineage marking mice will help determine the cellular origin of colon tumors and whether that is altered by apoptotic deficiency (44, 45).

Does apoptotic deficiency affect mutation spectrum and load?

The canonical Wnt pathway is invariably activated in human colon cancer due to mutations in

APC or β -catenin (46), while mutations in β -catenin are prevalent in AOM/DSS- or AOM-induced colon cancer in rodents (47). *PUMA*-deficient tumors acquire fewer activating β -catenin mutations compared to WT tumors and more aggressive behaviors (7), suggesting that a preexisting deficiency in apoptosis impacts on mutation spectrum or frequencies. Whole genome mutational analysis might help identify genetic alterations cooperating with apoptotic deficiency to promote oncogenesis, leading to a more dynamic view of mutation spectrum in carcinogenesis. Furthermore, similar studies coupled with isolated stem cells will make it possible to answer whether apoptosis affects mutation load in the stem cells to suppress tumor initiation, a long standing question in cancer biology. These types of experiments along with stepwise introduction of mutation, will help better model the multi-step process of carcinogenesis in mice.

Apoptosis as a link between cancer and inflammation

The AOM/DSS model involves both DNA damage and inflammation, important components in human colon cancer. Epidemiological studies have indicated a clear association between increased cancer risks and chronic inflammation and infections. The risk of developing colon cancer is drastically increased in patients with Crohn's disease or ulcerative colitis. The immune system plays a critical role in tumor promotion and control depending on the stage as well as type of tumors (48). But little is known how it affects tumor initiation. Is there any reason to believe that DNA damage response plays a role? The answer is likely to be yes.

First, immune cells produce various mutagens during inflammation including reactive oxygen and nitrogen species (RONS), which can damage DNA among other macromolecules in the epithelial cells (48). This is supported by a recent study using a similar AOM/DSS model, in which deleting an essential DNA glycosylase *Aag1* in the base excision repair (BER) pathway led to enhanced colonic tumorigenesis and increased mutations or base modifications (49). Second, inflammatory cytokines such as TNF- α can induce apoptosis of the intestinal epithelium partly through PUMA (50), which sets up an environment for selecting apoptosis-deficient cells. Third, chronic tissue repair and proliferation can overwhelm the endogenous repair capacity leading to fixed mutations. Together, a model is emerging in which defective apoptosis

promotes tumorigenesis by enhancing the survival of damaged epithelial cells. Such hypothesis is further supported by our observation that DSS induces PUMA expression in the colonic mucosa in the absence of p53 stabilization (Figure. 2). In addition, stresses such as ROS and ER stress (commonly associated with inflammatory responses) are found to induce PUMA, likely through transcription factors other than p53 (18, 51) (Figure. 3). It will certainly be interesting to clarify the role of PUMA-mediated tumor suppression in relation to p53 using inflammation-induced tumor models such as *IL-10* knockout mice or DSS treatment alone. Future work is needed to better understand the interplay between the epithelium and immune system for developing strategies for tumor prevention and treatment.

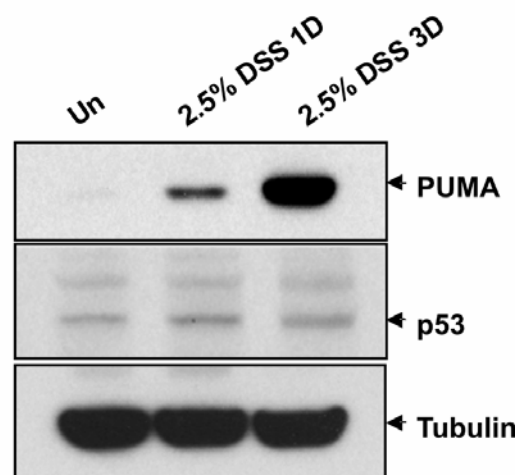


Figure 2. PUMA expression is induced by DSS in the colon. WT mice were subjected to DSS treatment in drinking water for the indicated time, D (day). The levels of PUMA, p53 and tubulin in the intestinal mucosa were analyzed by Western blotting.

Targeting apoptosis in cancer prevention and treatment

It is believed that the maintenance of tumor phenotypes is highly dependent on suppression of apoptosis triggered by so called “oncogenic stress”(3). The very same alterations leading to tumor initiation and progression contribute to therapeutic resistance (6, 52). Conventional chemotherapeutics and radiation primarily target rapidly dividing cells through apoptosis induction. Lack of PUMA induction leads to chemo or radio resistance, while elevated PUMA expression induces profound chemo or radio-sensitization of cancer cells (11, 12). The BH3-only proteins including Bim and PUMA have

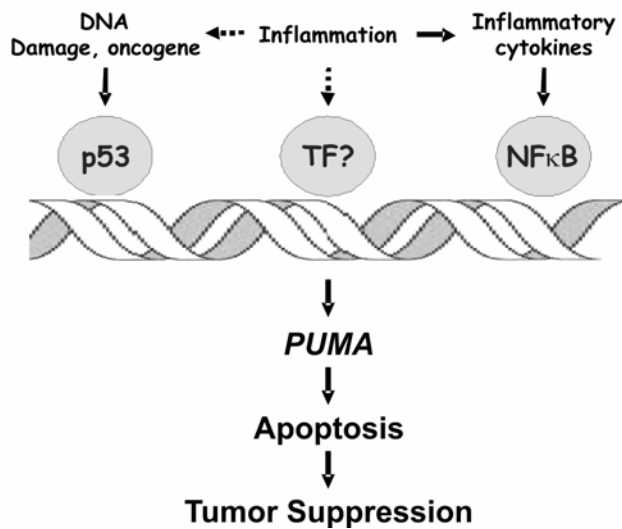


Figure 3. A model of PUMA-mediated tumor suppression. PUMA is activated by transcription factors in response to stress, leading to apoptosis induction and tumor suppression. DNA damage or activated oncogenes induces PUMA through p53 to promote apoptosis. Inflammatory cytokines induces PUMA through NFκB to promote apoptosis. DNA damage, inflammatory cytokines and perhaps yet-to-be identified mediators and transcription factors (TF) might provoke PUMA-dependent apoptosis during inflammation.

recently been shown to mediate apoptotic responses to agents targeting oncogenic kinase pathways in cancer cells (27, 53-56). Furthermore, chemopreventive agents such as nonsteroidal anti-inflammatory drugs (NSAIDs) can also promote apoptosis in colon cancer cells (20). Together, these observations strongly suggest that eliminating damaged or abnormal cells through PUMA-mediated apoptosis might be useful in preventing and treating cancer (Figure. 3). A growing number of small molecule BH3 mimetics have shown promise in preclinical studies and are now in various stages of clinical testing (24, 57). Obviously, apoptosis promoting agents might carry some risks. Too much of it can potentially lead to stem cell exhaustion and premature aging, which was modeled by the “super p53” mice that resist cancer development but age prematurely depending on the dosage and genetic background (58, 59). Cautions certainly need to be exercised to find a balance between tumor suppression and aging in cancer prevention or treatment. Losing a few more damaged stem cells is perhaps a price worth paying to stall cancer.

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Conflicts of Interest

No potential conflicts of interest to disclose.

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