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Curr Colorectal Cancer Rep. Author manuscript; available in PMC 2015 December 01.

Published in final edited form as:
Curr Colorectal Cancer Rep. 2014 December 1; 10(4): 411–416. doi:10.1007/s11888-014-0252-3.

Targeting Notch Signaling in Colorectal Cancer

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Abstract

The activation of Notch signaling is implicated in tumorigenesis in the colon due to the induction of pro-survival signaling in colonic epithelial cells. Chemoresistance is a major obstacle for treatment and for the complete eradication of colorectal cancer (CRC), hence, the inhibition of Notch is an attractive target for CRC and several groups are working to identify small molecules or monoclonal antibodies that inhibit Notch or its downstream events; however, toxicity profiles in normal cells and organs often impede the clinical translation of these molecules. Dietary agents have gained momentum for targeting several pro-survival signaling cascades, and recent studies demonstrated that agents that inhibit Notch signaling result in growth inhibition in preclinical models of CRC. In this review, we focus on the importance of Notch as a preventive and therapeutic target for colon cancer and on the effect of WA on this signaling pathway in the context of colon cancer.

Keywords

Notch-1; chemoresistance; dietary agent; prevention; treatment; colon cancer

Introduction

Cancer is a hyper-proliferative disease in which cells grow in an uncontrolled manner and acquire an invasive phenotype, leading to metastatic disease [1]. Worldwide, colorectal cancer (CRC) is the third most common malignancy in males and the second most common malignancy in females [2]. In the United States, CRC is the third leading cause of cancer-related deaths in men and women [3] and accounts for 136,830 new cases and 50,310 deaths annually. As per the National Institute of Health, total cost for the treatment of CRC for the year 2010 was fourteen billion dollars in United States. The mainstay of management of the early stage of CRC is still surgical resection with adjuvant therapy; the advanced stage disease may not be amenable to adjuvant modalities due to chemoresistance. Although early detection of CRC has improved, the mortality rate remains high due to chemoresistance and systemic toxicity to normal cells and organs [1].

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Conflict of Interest
Suman Suman, Trinath P. Das, Murali. K. Ankem, and Chendil Damodaran declare that they have no conflict of interest.

Compliance with Ethics Guidelines
Human and Animal Rights and Informed Consent
This article does not contain any studies with human or animal subjects performed by any of the authors.

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CRC is a multistep process that involves the disruption of molecular mechanisms necessary for intestinal homeostasis by maintaining intestinal proliferation, differentiation and programmed cell death. The repercussions of this deregulation of cellular signals promotes oncogenic phenotypes, in which cells exhibit uncontrolled proliferation, the loss of apoptosis, a highly invasive phenotype that advances to metastasis, the induction of angiogenesis and chemoresistance to drugs [4]. It is imperative to understand the molecular mechanisms involved in the development and progression of CRC to identify novel targets and develop novel drugs, which could benefit patients with CRC by avoiding the disadvantages associated with current treatment modalities. This review article focuses on the potential of the natural compound Withaferin A (WA) and its ability to target Notch signaling and impede CRC development and progression.

Notch signaling and components

The Notch signaling pathway consists of five Notch ligands (i.e., the Delta-like ligands DLL1, DLL3 and DLL4 and the Serrate-like ligands Jagged1 and Jagged2) and four Notch receptors (i.e., Notch1-4). Notch receptors are type-I transmembrane proteins with (i) an extracellular domain (NECD); (ii) a transmembrane domain; and (iii) an intracellular domain (NICD) that contains a RAM domain, six ankyrin repeats, and a transactivation domain [5, 6]. The activation of Notch depends on cell-to-cell contact in which Notch ligands present on a cell bind to Notch receptors present on a neighboring cell (i.e., the signal-receiving cell) [5, 7] and trigger metalloproteases in the a disintegrin and metalloprotease (ADAM) family, resulting in the cleavage of the extracellular domain [8]. Subsequently, activated γ-secretase cleaves within the transmembrane domain of Notch receptors, resulting in the release of the NICD [6, 9]. The NICD translocates to the nucleus and binds via its RAM and ankyrin domains to the DNA-binding transcription factor CSL (which consists of CBP or RBP-JK in vertebrates, Su (H) in Drosophila, and Lag-1 in *Caenorhabditis elegans*), Mastermind like-1 (MAML-1) and *p300/CBP*. Once formed, this complex displaces the co-repressors bound to the transcription factors, recruits transcriptional co-activators and induces the expression of target genes, such as hairy-enhancer-of-split (Hes-1) and Hes-related protein gene families [5, 6, 10, 11], that subsequently execute pro-survival functions.

Notch signaling and intestinal homeostasis

Notch signaling is required for the normal maintenance and homeostasis of the intestinal epithelium [5, 12, 13]. In particular, this pathway plays an important role in controlling the cellular fate of intestinal stem cells and the differentiation of colonic goblet cells [11, 14, 15]. The expression of components of this signaling pathway has been demonstrated in both the developing and adult intestine [16, 17]. Various studies demonstrated that the intestinal epithelium is enriched in the expression of Notch1, Notch2, DLL1, DLL4 and Jagged1 within the crypts. The secretory lineage of crypt cells, including the crypt base goblet cells in the colon, exhibit high levels of expression of DLL1 and DLL4 [18–20]. In the human colon, the Notch-1, -2 and -3 are highly expressed at the basal crypt while CSL and Jagged1 are highly expressed at the top of the crypts [21]. Notch signaling is crucial for the proliferation of crypt progenitors and for the differentiation of colonic epithelial cells [6].

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Published studies demonstrate that deleting CSL/RBP-Jκ in combination with the deletion of Notch1 and Notch2 or treatment with a γ-secretase inhibitor skewed colon-based columnar stem cells to differentiate into intestinal secretory cells, primarily goblet cells [15, 22]. Conversely, in transgenic mice, ectopic expression of the NICD throughout the intestinal epithelium caused a marked decrease in secretory cell production, indicating that Notch activation leads to the amplification of the intestinal progenitor pool and the inhibition of cell differentiation [11, 23]. In addition, Notch signaling is necessary for and works synergistically with Wnt signaling to promote the maintenance of the gut [24]. Notch and Wnt signaling act synergistically to inhibit the terminal differentiation of intestinal epithelial cells by downregulating the basic helix-loop-helix transcription factor ATOH1 (*Atonal homolog 1*), which is also termed MATH1 or HATH1 [25]. ATOH1 is a transcriptional activator that is repressed by HES-1; this protein is one of the most abundant targets of Notch and plays an opposing role to that of Notch/Hes by promoting secretory lineage differentiation [26–28].

The importance of Notch signaling in cancer

Notch signaling is critical for maintaining the balance between cell proliferation, differentiation, and apoptosis and is also involved in angiogenesis and the migration of cancer cells [6, 29]. Hence, deregulation of these processes that are regulated by Notch signaling may lead to the initiation and/or progression of CRC [30]. Notch receptors and their ligands are aberrantly activated in many human cancers, such as T-ALL [31, 32], pancreatic cancer [33, 34], breast cancer [35–37], prostate cancer [38–40], liver cancer [41], cervical cancer [42, 43], Kaposi's sarcoma [44], lung cancer [45], ovarian cancer [46], lymphoma [47], renal cancer [48] and CRC [49, 50]. Overexpression of Notch elements, such as receptors, ligands and downstream target genes, is correlated with increased progression, metastatic potential, and recurrence and poor prognosis and clinical outcome in various cancers [38]. For example, overexpression of Notch1 is associated with decreased time to recurrence in breast cancer [51]; similarly, high expression of Jagged-1 is correlated with the recurrence of prostate cancer [38]. Moreover, inhibiting Notch signaling with γ-secretase (GSI) in rodents caused a noticeable overproduction of goblet and enteroendocrine cells [52–54].

It was previously demonstrated that Notch is activated in primary CRC rather than metastatic colon cancer, implying that the activation of Notch may be an early step of CRC development [55]. In contrast, a more recent study reported high expression of Notch-1 and its target gene Hes-1 during both colon cancer progression [9] and metastasis [4]. No clear mechanism for the constitutive activation of Notch has been reported, and the implications of this activation for the initiation and progression of CRC remain unknown; however, mutations in the Notch receptor may play a significant role. In addition, the activity of Notch1 is also increased as a result of β-catenin-mediated upregulation of the Notch ligand Jagged-1 [56].

Notch ligand Jagged1 is highly confined to enteroendocrine cells and is undetectable in the mucosa of the small or large intestine; however, higher expression of this ligand is observed in human colon tumors (12–20). A recent study reported that downregulation of Jagged1

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decreases cell viability and causes cell cycle arrest by downregulating the expression of Cyclin D1, Cyclin E and c-Myc in CRC [50]. These in vitro studies also demonstrated a reduction of the migratory and invasive behavior of CRC cells. Further, knocking down Jagged-1 inhibited the growth of xenograft tumors compared to controls, supporting the therapeutic role of Notch in CRC models [50]. Activation of Notch signaling has been reported to be indispensable for the development of adenomas in $APC^{Min/+}$ mice and for the self-renewal of tumor-initiating cells [1].

Targeting Notch signaling in CRC

Various approaches are being used to inhibit Notch signaling and are under investigation in many cancer types; this topic is discussed in detail by Espinoza and Miele [57]. These approaches include neutralizing Notch antibody, in which blocking monoclonal antibodies (mAb) are directed against Notch receptors (i.e., Notch-1, -2, -3, -4). In addition, blocking antibodies against Notch ligands are under development. A novel mAb against the extracellular domain of nicastrin has also been generated [58]. This mAb recognizes fully mature nicastrin in the active γ-secretase complex and inhibits its activity. Another attractive therapeutic candidate is decoy, which is the soluble form of the extracellular domains of Notch receptors [59]. These decoys compete with their cell surface-bound endogenous counterparts and abolish Notch signaling, as they lack the transmembrane region that is necessary for receptor activation. In another approach, various clinical trials have focused on blocking the cleavage process of Notch receptors with γ-secretase inhibitors (GSIs) [60, 61]. γ-secretase is a promising target for Notch inhibition and exhibits cytostatic or cytotoxic activities in various cancer cells [57]. Silencing Notch1 with GSIs sensitizes colon cancer cells to chemotherapy [9, 61]. Although GSIs appear to be attractive tools for inhibiting Notch signaling, there are some drawbacks associated with these inhibitors, as they exhibit some off-target and adverse effects, leading to gastrointestinal toxicity and liver injury [50, 62]. Another study published by Timme et al. demonstrated the nonspecific effects of a GSI [63]. GSIs are known to induce apoptosis while enhancing the response to chemotherapy in various cancers. However, in this study, the authors found that treating colon cancer cells with a potent inhibitor of γ-secretase reduced oxaliplatin-induced apoptosis by increasing the expression of anti-apoptotic proteins (i.e., Mcl-1 and/or Bcl-xL). In addition, GSI treatment alone exerted no apoptosis or growth inhibitory effect. This study is surprising and demonstrates that caution is warranted when treating colon cancer with GSIs in combination with chemotherapeutic agents.

Although many approaches are available to inhibit Notch signaling, these approaches are either in their infancy or are under investigation due to toxicity issues. Thus, it is very important to identify small molecules or establish regimens that target Notch signaling in CRC with minimal or no side effects. One such approach is the use of dietary chemopreventive agents, which might be non-toxic to normal cells/organs while selectively inhibiting tumorigenesis [64–68]. Natural products are of paramount importance for the identification of novel anticancer agents. One such plant-derived natural product is WA. This product has gained considerable scientific attention. The next section of this article will focus on WA and its anticancer properties and effects.

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Withaferin A targets Notch signaling

Withaferin A [(4β,5β,6β,22R)-4,27-dihydroxy-5,6:22,26-diepoxyergosta-2,24-diene-1,26-dione)] is a bioactive compound derived from the medicinal plant *Withania somnifera* Dunal. This plant is commonly known as Ashwagandha, Indian ginseng or Indian winter cherry. *Withania somnifera* is a small subtropical shrub, and products from this plant have been used safely for centuries in the Indian Ayurvedic system of medicine to treat various ailments and to increase longevity and vitality [68–73]. The root and leaf extracts of this plant protect against chemical-induced carcinogenesis in experimental rodents, as reviewed previously [74]. WA is a steroidal lactone and is derived from the leaves and roots of this plant [75]. The ethnobotanical history of WA-containing herbal preparations reveals the numerous effects of this compound, including anticancer and anti-inflammatory effects [76], a preventive role in neurologic disorders [69] and growth inhibitory properties in various cancer cell lines, including human colon cancer cells [77, 78]. This natural compound WA exhibits diverse pharmacologic activities (Figure 1), including anti-inflammatory, cardioactive, central nervous system, immunomodulatory and antiangiogenic effects [79–81]. WA also exerts analgesic and antipyretic effects in mice [82]. WA targets vimentin in soft tissue sarcoma, suggesting a role of this compound in the modulation of epithelial to mesenchymal transitions [83].

In recent years, our laboratory explored the anticancer effects of WA on various cancer types and demonstrated the incredible potential of this natural compound as an anticancer agent [84–86]. Our results revealed an inhibition of Notch1 signaling in WA-treated CRC at both the RNA and protein levels. Notch1 inhibition also affected downstream targets, such as Hes-1 and Hey-1. Interestingly, no inhibitory effects on γ-secretase complex subunits were observed. This WA-mediated inhibition of Notch1 also affected other pro-survival signaling pathways, which are reported to be involved in crosstalk with Notch signaling. WA inhibited the AKT/NF-κB/Bcl-2 axis, thereby inhibiting cellular proliferation and inducing apoptosis in these colon cancer cells. In addition, we also observed a concomitant downregulation of mTOR signaling in WA-treated CRC cells.

The overexpression of Notch1 in colon cancer cells increased the expression of the downstream targets Hes-1 and Hey-1. In addition, this overexpression resulted in increased expression of pAKT and the mTOR signaling components pp70S6K and p-4E-BP1. In contrast, knockdown of Notch1 had the opposite effect, confirming Notch-mediated modulation of Akt and mTOR signaling. These results suggest that Notch is upstream of Akt and mTOR signaling. In addition, Notch signaling interacts with the ERK and JNK pathways. Notch negatively regulates c-Jun and JNK in various cancer types. In this study, WA treatment also upregulated phosphorylated c-Jun expression and JNK expression, thereby inducing apoptosis in colon cancer cells [84]. Similar findings were observed in breast cancer, where WA resulted in the inhibition of Notch1 [87]. In ovarian cancer [88] cells, WA downregulated expression of Notch1 and Notch3 was observed. WA treatment of CaOV3 and SKOV3 ovarian cancer cells inhibited the growth and colony formation efficiency of these cells [88]. WA also induced cell cycle arrest and apoptosis in these cancer cells. At the molecular level, these changes were accompanied by the downregulation of the Notch1, Notch3, cdc25C, total Akt, phosphorylated Akt and bcl-2 proteins. This study

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suggests that WA is a potential therapeutic agent for ovarian cancer. Consistent with these studies that investigated WA and Notch signaling, our unpublished data demonstrate that WA inhibits tumor growth in xenograft models of colon cancer.

Conclusions and future directions

Notch signaling is an important therapeutic target, as it plays a major role in the colonic crypt compartment by maintaining colon homeostasis via the regulation of colon stem cell behavior and differentiation. However, aberrant activation of Notch initiates and promotes colon carcinogenesis, hence selectively targeting Notch signaling in colon cancer cells could be an ideal strategy for prevention and treatment of colon cancer. WA appears to have tremendous anticancer potential as a result of targeting multiple molecules in a variety of human cancers, including CRC, as evidenced by various preclinical in vitro and in vivo studies. Only our group has demonstrated the Notch inhibitory role of WA in CRC. It will be interesting to study the effect of this molecule in colon cancer stem cells that reside in the colon crypts and play an important role in this disease. In addition, the effect of this natural compound in combination with current chemotherapeutic drugs must be studied. We are currently generating potent WA analogs, which could be even more effective in inhibiting Notch signaling than WA. Appropriately powered studies are needed to take this molecule, which has been known to have therapeutic value for decades, from bench to bedside in the clinical setting.

Acknowledgments

This study was supported by NIH Grant 1R01CA185972-01.

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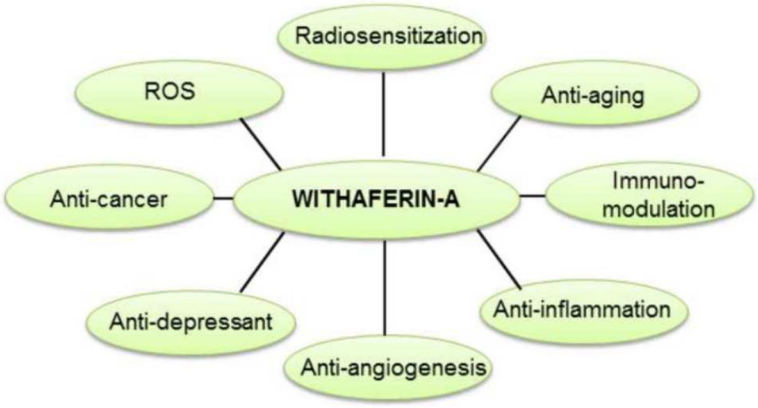

Figure 1.
Medicinal properties of Withaferin-A