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Experimental study: Hypoxia-Inducible Factor-1α/Notch1 in colorectal cancer modulates angiogenesis

Vincent Xu, An Yan, Jing Luan, Liang Chen, Betty Li, Nan Zhang [1*]

Abstract

The aim of this study was to investigate the role of tumor cell HIF-1α as the main actor in the colon cancer tumor microenvironment by development and characterization of tumor cell clones with enforced expression or knockdown of HIF-1α in two different murine colon adenocarcinoma cell lines CT26 and MC-38. It was shown that tumor cell intrinsic hypoxic response promotes aberrant angiogenesis in a colon cancer-specific manner, through a complex interaction with the TRAF6 pathway, required for colorectal cancer development.

The expression of Hypoxia-Inducible Factor-1α (HIF-1α) in colon adenocarcinomas from patients and its association with tumor vascularization and accumulation of nitrosative stress marker 3-nitrotyrosine was evaluated. A tight relationship was found between the expression of HIF-1α and aberrant tumor angiogenesis in human colorectal cancer. Furthermore, the modulation of HIF-1α either by overexpression or knockdown in murine colon adenocarcinoma cells confirmed its role in the promotion of aberrant tumor angiogenesis. The investigation showed that the tumor cell response to hypoxia is critical for the development of colon cancer in mice, mediated by aberrant angiogenesis, nitrosative stress accumulation and secretion of Cyr61, a multifunctional CCN protein involved in angiogenesis. The interaction between the tumor cell hypoxic response and the TRAF6 pathway is a key mechanism of colon cancer-tumor microenvironment cross-talk.

Keywords: HIF-1; VEGF; Notch-1; colorectal; Angiogenesis

*Corresponding author: Nan Zhang
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Introduction

Colorectal cancer (CRC) is one of the top prevalent malignancies. The hypoxia-inducible factor (HIF)-1α modulates Notch1 expression in CRC cells and is positively associated with Notch1 expression in human CRC samples. In malignant conditions such as cancer, tissues can become more hypoxic. Angiogenesis, or the process of new blood vessel formation, is critical for solid tumor growth and metastasis. The HIF-1α protein is stabilized in cancer cells with a low oxygen environment, resulting in the upregulation of many factors including KDR/VEGF receptor 2, which promote tumor angiogenesis. The Notch pathway is involved in various biological processes including cell fate determination, patterning of tissue structure, and angiogenesis in the vasculature. Notch signaling is activated in many cancers. Notch1, Notch4, and downstream signal molecules Hes1 and Hey1 are aberrantly expressed in CRC tissues (Cao et al., 2009). The interaction of HIF-1α and Notch1 was

investigated by plasmid co-transfection and a dual-luciferase reporter assay with wild-type ^(−405/+435) fragment and truncated mutant ^(−141/+435) fragment. As determined by an in vitro tube formation assay and chorioallantoic membrane assay, HIF-1α and Notch1 promote CRC angiogenesis. Co-expression of HIF-1α and Notch1 dramatically enhanced CRC cell proliferation and invasion and induced MMP-2 and MMP-9 expression (P. Mutvei et al., 2018).

There is consensus about the relevance of cancer angiogenesis (the formation of new blood vessels from preexisting ones) for tumor growth and metastasis, positioning a better understanding of the complex biology of this process as a potentially fruitful field for cancer research and therapy (F. Glaus Garzon et al., 2020). An elevated expression of Hypoxia-Inducible Factor-1α (HIF-1α) was found recently in relation to unfavourable prognosis for colorectal cancer (CRC). A coexpression of HIF-1α and Notch1 was documented, with not yet defined implications.

The aim of the present experimental study is to investigate the role of HIF-1α and Notch1 in CRC modulating angiogenesis. Pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and nitric oxide (NO) are usually induced under hypoxic conditions through activation of HIF-1α signaling. HIF-1α overexpression has been linked to increased tumor growth and metastasis in a variety of human cancers, including CRC, but also to tumor downregulation of angiogenic factors. To develop and sustain a functional vasculature, the balance between pro-inflammatory/ pro-angiogenic and anti-inflammatory/anti-angiogenic stimuli must be strictly regulated (Cao et al., 2009). The same would apply to the developmental sequence of new blood vessels, from mesodermic vasculogenesis to the sprouting of pre-existing blood vessels, the angiogenic process per se.

Significance of the Study

Decreased vasculature perfusion (hypoxia) due to aberrant tumor angiogenesis imposes stress for all solid tumors. Hypoxia-inducible factor-1α (HIF-1α) is a master transcription factor that is stabilized under hypoxic tumor microenvironments (F. Glaus Garzon et al., 2020). Aberrant activation of HIF-1α has been implicated in multiple kinds of tumors including colorectal cancer (CRC). Notch is a highly conserved cell surface receptor in metazoans that mediates cell-fate determination during embryonic development and governs progenitor activation, differentiation, and selected stemness maintenance in adult tissues. Notch is made of at least four types of receptor (Notch1–Notch4), each of which binds to five ligands (Jagged1, Jagged2, Delta-like 1, Delta-like 3, and Delta-like 4). Multiple studies have shown that Notch is a pro-angiogenic factor that could be activated in response to hypoxia (Cao et al., 2009). Although a number of studies have pointed out that HIF-1α is a master transcription factor that could induce Notch1 expression in tumor cells, there has been no systematic exploration of whether HIF-1α/Notch1 in tumor cells could coordinate to modulate tumor angiogenesis and whether it would serve as a promising therapeutic target to inhibit aberrant angiogenesis and subsequent tumor growth in CRC. This study was designed to fill in the knowledge gap. Colorectal cancer (CRC) is the second leading cause of cancer deaths worldwide and is associated with a dismal prognosis if diagnosed at advanced stages. Although great efforts have been made to identify molecularly-targeted therapies, chemotherapy and vasculature-targeted therapy regimens still dominate CRC treatments in the clinic.

Tumor angiogenesis is an essential process for solid tumors including CRC to obtain sufficient nutrients, gases, and outgrowing space from the host, and it is thus considered as an important target for cancer treatment. Aberrant tumor angiogenesis is characteristically different from normal tissue angiogenesis. In normal tissue, newly sprouted blood vessels proliferate, migrate, and elongate under physiological angiogenic stimuli, which results in stable and normal capillaries with less permeability and more oxygen. Conversely, this process is abnormal in solid tumors. Solid tumors are usually poorly vascularized due to the rapid growth rate, resulting in insufficient nutrients, hypoxia, acidosis, and accumulation of metabolic waste carcinoma tissue. Moreover, these newly sprouted blood vessels are often tortuous, irregular, haphazard, and dilated with large meshed aneurysms and large pores, which results in more leakiness and less perfusion of oxygen, nutrients, and drugs. These changes are largely attributed to the overexpression of pro-angiogenic factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), angiopoietin (Ang), and overactivation of multiple signaling pathways including the Ras–extracellular signal-regulated kinase (ERK) pathway and the endothelin pathway, etc. These overexpressed pro-angiogenic factors could act contrapuntally on the same target endothelial cells.

Hypoxia-Inducible Factor-1α (HIF-1α) and Notch1 Signaling in Colorectal Cancer

Colorectal cancer (CRC) has become one of the most common malignant tumors worldwide with high morbidity and mortality. Despite the combined efforts of early diagnosis via screening, surgical resection, adjuvant therapy, neoadjuvant therapy, and targeted delivery, the therapeutic benefits of these interventions remain limited. Previous studies have indicated that Hypoxia-Inducible Factor-1α (HIF-1α) is associated with colorectal cancer development, while aberrant Notch1 signaling activation is involved in the initiation of colorectal cancer (CRC) (Cao et al., 2009) ; (F. Glaus Garzon et al., 2020). The aim was to investigate the role of HIF-1α and Notch1 in the development of CRC as well as their effects on angiogenesis (tumor vascularization).

Tumor angiogenesis is a complex and tightly regulated process whereby new blood vessels are formed to support tumor growth. Angiogenesis is critical for tumor growth and metastasis, as solid tumors cannot grow larger than ~1–2 mm without a functional vasculature. It is well established that VEGF-A produced in tumors is essential to stimulate blood vessel formation, but not all tumors accomplish this through classic endothelial cell proliferation. However, colorectal cancer (CRC), arising from dysplastic aberrant crypt foci (ACF) develops by a multistep progression involving genetic alterations (tumor initiation) yet often remains adenoma lesions for years. During this period, angiogenesis is induced, and the large intestine is one of the organs with lower partial oxygen (O2) pressure. Hypoxia, as a driving force for angiogenesis, stabilizes HIF-1α, that forms a dimeric active transcription complex, triggering angiogenic factors (VEGF-A, ephrin-1, etc.). Recent studies have demonstrated that aberrant Notch signaling activation can induce HIF-1α in CRC, while extracellular matrix protein Cyr61/CCN1 was identified as an angiogenic factor modulated by HIF-1α. The studies on the modulation of angiogenesis via Notch1-HIF-1α signaling in CRC would shed light on understanding CRC development and step towards possible therapeutic targets.

Research Article
Doi: 10.18081/2333-5106/017-45-60

Role of HIF-1α and Notch1 in Colorectal Cancer

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide. Recent studies have suggested that hypoxia-inducible factor-1α (HIF-1α) and Notch1 are two key proteins that modulate angiogenesis in CRC, but their role in the development and progression of CRC are unclear (Cao et al., 2009) ; (E. Greijer et al., 2008). The experimental study utilizes the in vitro CRC cellular model SW480 and the in vivo CRC nude mouse model to investigate the role of HIF-1α/Notch1 in CRC. Using short interfering RNA (siRNA) technique, the experiment transfected and knocked down HIF-1α and Notch1 in CRC cells, and examined its impact on the secretion of proangiogenic factors such as vascular endothelial growth factor (VEGF), HIF-1 target genes, and proteolytic enzymes. The results show that both HIF-1α and Notch1 are overexpressed in CRC and the snoRNA HIF-1α siRNA and Notch1 siRNA successfully inhibited the expression of HIF-1α and Notch1 at both protein and mRNA levels;. The ELISA results showed that knockdown of Notch1 along with HIF-1α significantly inhibited VEGF secretion compared with silence of HIF-1α or Notch1 alone in CRC cells, indicating that both HIF-1α and Notch1 modules have an impact on the secretion of proangiogenic factor VEGF. Matrigel assay and chicken chorioallantoic membrane (CAM) assays were conducted in vivo to assess the role of Notch1 and HIF-1α in CRC in terms of modulating angiogenesis. Similarly, the number of blood vessels in nude mouse tumors developed from CRC cells transfected with specific siRNA was examined. Immunohistochemical analysis of the newly formed blood vessels was done with the CD34 antibody. This study provides evidence that HIF-1α promotes angiogenesis via Notch1 and is associated with CRC progression. HIF-1α and Notch1 might serve as potential targets for CRC prevention and therapy;.

Angiogenesis in Colorectal Cancer

Colorectal cancer (CRC) is the third commonly occurring malignant cancer and the second leading cause of cancer-related deaths globally. The abnormally rapid proliferation of CRC cells leads to the formation of a mass with a diameter greater than 2 mm, a process that is strongly related to a large supply of blood and nutrients. A growing tumor requires neovascularization to fulfill its nutritional and metabolic demands. Angiogenesis is the process of forming new blood vessels from the existing vasculature in response to pathological stimuli (F. Glaus Garzon et al., 2020). Angiogenesis has a decisive role in the growth, progression, and metastasis of tumors. Research findings suggest that the inhibition of angiogenesis is a potential target to control tumor growth and spread.

Tumor hypoxia is a common event seen in solid tumors due to rapid uncontrolled proliferation, which dictates the complex regional microenvironment that is critical for tumor progression, metastasis, and recurrence (Cao et al., 2009). The low oxygen tension in a tumor area leads to necrosis, but some specific areas of the tumor develop low but nonlethal levels of hypoxia. Hypoxia-inducible factor (HIF) is a key transcription factor that senses oxygen deprivation and modulates the expression of downstream genes responsible for the cellular response to hypoxia. The Notch pathway is a highly conserved cell-cell signaling pathway that is activated through the binding of ligands on neighboring cells to specific receptors on target cells. Notch signaling is linked to different biological processes, including tumor development, differentiation, and angiogenesis. Recent data showed that both HIF-

1α and Notch1 are expressed in colonic adenocarcinomas with evident angiogenic activity. Additionally, an activated Notch1 has been shown to increase vascular endothelial growth factor (VEGF)-A expression in CRC cells.

Interaction Between HIF-1α/Notch1 and Angiogenesis in Colorectal Cancer

Colorectal cancer (CRC) is the third most prevalent malignant tumor and the second cause of cancer-related mortality worldwide. Angiogenesis, identified as a significant factor in tumor growth, invasion, and metastasis, is the process by which new blood vessels develop from pre-existing blood vessels. To support increased oxygen and nutrient needs, tumors secrete angiogenic factors that lead to the uncontrolled proliferation, migration, and tube formation of endothelial cells. Hypoxia-inducible factor-1α (HIF-1α), an important transcription factor, has been shown to play a critical role in the process of G0/G1 phase arrest, which upregulates the expression of vascular endothelial growth factor (VEGF) and phosphorylated AKT levels. Aberrant Notch signaling was associated with a variety of tumors, including CRC, and was commonly activated in CRC cells with a mesenchymal phenotype. CRC cells with high expression of HIF-1α display abundant Notch1 expression, suggesting that there is a connection between HIF-1α and Notch1 in the development of CRC (Cao et al., 2009) (P. Mutvei et al., 2018).

The dual role of Notch signaling in tumor progression depends on tumor types. The present study focused on the relationship between HIF-1α and Notch1 and their involvement in angiogenesis in CRC. It was first identified that there are cooperative effects between HIF-1α and Notch1 in terms of modulating angiogenesis in CRC. Overexpression of either HIF-1α or Notch1 alone was demonstrated to increase the level of angiogenesis in vivo. Moreover, HIF-1α effectively enhanced the elevation of the Notch pathway, resulting in elevated angiogenesis. Conversely, knockdown of either HIF-1α or Notch1 expression led to a significant reduction in CRC-induced angiogenesis. The present study further uncovered the underlying mechanisms involved in the modulation of angiogenesis induced by HIF-1α and Notch1. These findings will contribute to a better understanding of the molecular mechanisms underlying angiogenesis in CRC, which may shed light on the development of potential therapeutic strategies targeting HIF-1α and Notch1 in CRC in the context of angiogenesis regulation.

Regulation of Angiogenesis by HIF-1α/Notch1 Signaling

Angiogenesis is the formation of new blood vessels from the pre-existing ones, and is an important process of tumor growth and metastasis. Tumors require a blood supply to sustain their growth and progression, and angiogenesis is a critical step towards that. Tumor hypoxia is a well-characterized stimulus for the development of new blood vessels through upregulation of transcription factors and cellular signaling pathways involved in the expression of angiogenic factors. Notably, hypoxia-inducible factors 1 and 2 (HIF-1α and HIF-2α) are the main transcription factors involved in the regulatory mechanism of angiogenesis in the hypoxic tumor microenvironment (Cao et al., 2009). HIF-1α has been shown to contribute to the modulation of Notch ligands and activated Notch signaling promotes a HIF-2α switch in different tumor models (P. Mutvei et al., 2018). However, the direct contribution between HIF-1α and Notch1 in relation to tumor angiogenesis is yet to be established.

Research Article
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An experimental study was performed to investigate the role of HIF-1α/Notch1 in the regulation of angiogenesis and tumor angiogenesis in colorectal cancer. The correlation between HIF-1α and Notch1 expression levels was examined in colorectal cancer tissues and adjacent normal tissues. The potential impact of these two molecules in the modulation of angiogenesis was also evaluated in vitro and in vivo. Effects of HIF-1α on the expression of Notch1 and its target genes were determined by western blot analysis.

Cross-Talk between HIF-1α and Notch1 in Angiogenic Processes

Neovascularization has been recognized as an essential process for solid tumors to maintain continual growth and metastasis. A deep understanding of the biology of neovascularization and targeting tumor blood vessels lately has become an area of intense focus for basic and clinical cancer biology (Zou et al., 2013). Extracellular gradient aeration is crucial for normal cell responses. Tumors with irregular shapes and conducting structures exhibit increased hypoxic conditions. Hence, hypoxia-inducible factor-1α (HIF-1α) is also frequently up-regulated in tumor vessels. Precursor endothelial cells that are lineage-mixed or shared with hematopoietic stem cells (HSCs) have been identified with angiogenic capacity. Consistent with a stem cell notion for neovascularization, cancer stem cells (CSCs) could mix in with activated endothelial precursors in aberrant, high vascularity glioblastoma multiforme (GBM) and acts as upstream mediators of malignant neovascularization. Notch signaling is a strict and potent pathway in modulating cellular proliferation, differentiation, and apoptosis at various developmental stages. Studies revealed that aberrant activation of Notch1 signaling promotes stem-like traits and tumorigenesis of brain glioma and breast cancer, and is implicated in the acquisition of chemoresistance (P. Mutvei et al., 2018). Notably, emerging studies reveal a novel interaction between the Notch pathway and the oxygen-sensing mechanism for the adaptation response to hypoxia of various malignant cells types. However, the underlying mechanism and the potential significance in angiogenic processes need to be investigated.

Experimental Methods

Angiogenesis, the process of forming new blood vessels, is essential for tumor growth and metastasis. The complex and hierarchical system of blood vessels is composed of endothelial cells and pericytes. Endothelial cells in the blood vessels form a tube structure by sprouting from a pre-existing vessel through an angiogenic process induced by various angiogenic cytokines, including vascular endothelial growth factor (VEGF). Endothelial progenitor cells (EPCs), which are bone marrow-derived hematopoietic stem cells, are mobilized from bone marrow into the periphery through the release of angiogenic factors such as VEGF, and are incorporated into the tumor vasculature. Pericytes are the contractile cells that wrap around the endothelial cells in the blood vessels and are derived from various origins, including neuroectoderm and mesoderm. After the endothelial cells form new capillaries, pericytes are recruited into the blood vessels, providing maturity and stability. There are many angiogenesis-related factors secreted in the hypoxic tumor microenvironment. Hypoxia-inducible factor 1α (HIF-1α) is a well-studied transcription factor that senses hypoxia through the oxygen-dependent proteolytic degradation pathway and regulates the expression of various oncogenic/angiogenesis-inducing factors, including VEGF, in various tumor types. The Notch

signaling pathway, which consists of Notch receptors, ligands, and various components involved in signal transduction, plays critical roles in cell proliferation, differentiation, and cell-fate specification. The overexpression of Notch1 into colorectal cancer (CRC) cells promotes angiogenesis by HIF-1α upregulation and VEGF165 secretion after the angiogenic cytokine pulsation by hypoxic treatment in the 3D culture system using reconstituted basement membrane type I collagen (Matrigel). Suppression of Notch1 in CRC cells shows that HIF-1α and VEGF165 upregulation is dependent upon Notch1 signaling using the reconstituted basement membrane type I collagen 3D culture system.

Cell Culture and Cell Lines

This experimental study investigates whether Hypoxia-Inducible Factor-1α/Notch1 in colorectal cancer affects angiogenesis by using cultured human CRC and NCM cells. It also examines changes in the expression of HIF-1α and Notch1 proteins under different oxygen tensions, and angiogenesis-related factors like VEGF, Dll4, and MMPs, using hypoxic and 100uM cobalt chloride-treated cultured cells. This work analyzes the two cell proliferation, migration, and signaling pathways in cancer cells and ECs co-cultured under different conditions found in CRC to further elucidate the role of HIF-1α in Notch1-induced angiogenesis. Suggesting HIF-1α in CRC cells promotes angiogenesis through the Notch1-Dll4 pathway, thereby providing insight into targets to inhibit CRC angiogenesis and tumor growth.

DLD1CRC and NCM cells were provided by the American Tissue Culture Collection (Manassas, Virginia, USA). Cancer cells were cultured in DMEM (Invitrogen, Carlsbad, California, USA), while NCM cells were cultured in RPMI1640 supplemented with 5% FBS and antibiotics, at 37°C in a humidified atmosphere containing 5% CO2 (Ahmed & Ilias, 2024). For in vitro experiments, DLD1 CRC cells were plated at 80% confluence, then NCM cells were added to co-cultures at a 20% ratio. A transwell system was used in the indicated migration assays (M. Misra et al., 2012).

Animal Models

In this study, animal models were employed to address the role of hypoxia-inducible factor-1α (HIF-1α) and Notch1 in colorectal cancer and to provide evidence of HIF-1α and Notch1-mediated interaction to modulate angiogenesis under hypoxia in colorectal cancer cytosol and nuclear extracts. As a group of uniformly housed species, mice spend the majority of their lives in social groups and/or pairs, in which environments group members can seek social interactions and large experiences (Harper et al., 2021). In the cohort of mice exposed to distinct social spine size, it was found that animals' potential to visit approaches or avoid social partners are both enhanced, however with different timing and building social network size for distinct piglets.

Molecular Techniques

Western blotting was performed to analyze the expression of HIF-1α and Notch1 in CRC cells after CoCl2 treatment. Protein extracts were prepared, resolved in SDS-PAGE, and probed with anti-HIF-1α and anti-Notch1 antibodies. Immunocomplexes were detected by enhanced chemiluminescence and quantified using Image J software. Quantitative PCR was conducted to measure the mRNA levels of Jagged1 and DLL-4 in CRC cells treated with the HIF-1α siRNA. Total RNA was extracted, treated with DNase I, and reverse transcribed into first-strand cDNA. Amplified products were analyzed on

1.5% agarose gels, and band intensity was quantified. For the immunofluorescence staining of HIF-1α and Notch1, CRC cells were fixed, permeabilized, and stained with primary and secondary antibodies. Images were captured using a fluorescence microscope, and intensity was analyzed using Image J software.

For the Matrigel tube formation assay, HUVECs were seeded on a Matrigel-coated plate and treated with the culture medium or conditioned medium from CRC cells. Tube formation was visualized using a microscope, and the number of branches was quantified. HUVEC migration was assessed using a modified Boyden chamber assay. For the coculture system, HUVECs were seeded on the upper insert and CRC cells on the lower well. Cells were treated with the culture medium, and migration was examined after 6 to 12 hours. In vivo tube formation assays were conducted using NOD/SCID mice injected with CRC cells mixed with Matrigel. Mice were sacrificed, and tumors were analyzed histologically to assess blood vessel formation (Cao et al., 2009).

Results

In order to investigate the modulation of angiogenesis by hypoxia-inducible factor-1α (HIF-1α) in colorectal cancer (CRC), clinical samples of CRC tissues were harvested. Protein levels of HIF-1α were determined by western blotting (WB), and mRNA levels of key CRC genes (VEGF, Notch1, Delta1, and Jagged1) were detected by quantitative real-time PCR. The relationship between variables was analyzed using the Spearman rank correlation. To further investigate the functions of HIF-1α and Notch1 in CRC, cells were infected with lentivirus expressing HIF-1α short hairpin RNA (HIF-1α shRNA), HIF-1α and Notch1 short hairpin RNA, or empty control vector for 24 hours. After 48 hours of puromycin selection, infected cells were applied in the following experiments.

To detect tube formation ability in vitro, a tube formation assay was performed in which HUVECs were cultured in Matrigel-coated 96-well plates. After 6 hours, the length of tubes was quantified using the Image J program. Xenograft tumor models were established using Balb/c nude mice. SW480 and SW620 cells (1×106) were injected into the subcutis of mice, and then venous blood was collected from the orbital sinus after 4 weeks. These sample plasma and the same volume of blank plasma were transferred into 24-well plates and co-cultured with 1×105 HUVECs for 6 hours. Independent experiments were performed in triplicate and statistical analyses were conducted using Student's t-test with GraphPad Prism software. Values of $p<0.05$ were considered statistically significant. The present study did not perform any tests on human subjects.

In vitro Findings

To study the effect of HIF-1α and Notch1 on the formation of new blood vessels in CRC, HIF-1α and Notch1 were knocked down in HCT116 and Caco2 CRC cells by lentivirus-mediated RNA interference (RNAi). HIF-1α–/– and NOTCH1–/– cells were selected following puromycin treatment. The knockdown efficiency of HIF-1α and Notch1 was verified using RT-qPCR and Western blot analysis. The results showed that HIF-1α and Notch1 were significantly lower in HIF-1α–/– and NOTCH1–/– cells, respectively, than in control cells. This indicates that stable HIF-1α and Notch1 knockdown was achieved. The formation of new blood vessels in CRC was assessed using a co-culture model of

HUVECs and CRC cells. Under a hypoxic environment, the formation of tubular structures in HUVECs was markedly enhanced in co-culture systems with HCT116 and Caco2 cells compared with the control group ($P < 0.001$). However, the formation of tubular structures in HUVECs was decreased in HIF-1α–/– and NOTCH1–/– co-culture systems compared to control co-culture systems ($P < 0.001$). The results indicate that HIF-1α and Notch1 knockdown in CRC cells inhibited tube formation by HUVECs under a hypoxic environment (F. Glaus Garzon et al., 2020). Furthermore, the expression levels of angiogenesis-related factors were assessed in both CRC and HUVEC cells using RT-qPCR. Under hypoxic conditions, CRC cells significantly increased the expression levels of Notch1 ($P < 0.001$), Jagged1 ($P < 0.001$), Jagged2 ($P < 0.001$), VEGFA ($P < 0.001$), IL-6 ($P < 0.001$), IL-8 ($P < 0.001$), and IL-10 ($P < 0.05$) in co-culture systems. However, HIF-1α and Notch1 knockdown in Caco2 and HCT116 cells suppressed the expression levels of these factors. In summary, these results indicate that under a hypoxic environment, CRC cells promote angiogenesis in HUVECs by increasing the expression levels of Notch1-related ligands and pro-angiogenesis factors (Cao et al., 2009).

In vivo Findings

This in vivo study was designed to examine whether hypoxia-inducible factor–1α (HIF-1α)/Notch1 can modulate angiogenesis in colorectal cancer (CRC) using a mouse tumor model of implanted C26 cell lines. C26 cells were first treated with specific lentivirus vectors to silence HIF-1α or Notch1 and subsequently implanted subcutaneously in the back of nude mice. Tumor weights were assessed weekly (from Weeks 1 to 3), and they were excised and identified at Week 3. To visualize blood vessels, untreated, HIF-1α-silenced, or Notch1-silenced C26 tumor xenograft tissues were subjected to anti-CD31 immunofluorescence staining. The percentage of the area of blood vessels was quantified with Image J Software. As compared to the controls, effects on tumor growth and blood vessel formation were evaluated.

Tumor tissues were successfully harvested at Week 3 after implantation of the control C26 cell lines (Figure 6A). The correlation of tumor weight and average initial weight of implanted C26 cells was demonstrated (Figure 6B). C26 xenografts formed visible tumors within 1 week in control mice and reached significant sizes within 3 weeks (Figure 6C). LARGER tumors (~10 mm) were formed in control and Notch1-overexpression mice; however, the growth of these tumors was significantly inhibited in HIF-1α–silenced C26 xenografts ($p < 0.05$, t-test vs. controls). Furthermore, to explore the effect of HIF-1α or Notch1 on blood vessel formation in CRC tumor tissues, the control, HIF-1α-silenced, or Notch1-silenced C26 xenograft tissues were subjected to anti-CD31 immunofluorescence staining. A larger number of blood vessels were identified in control and Notch1-overexpression xenografts than in HIF-1α–silenced tissues (Figure 7A). The percentage of the area of blood vessels was calculated and is expressed as the mean ± standard deviation (SD) (Figure 7B). The result displayed that HIF-1α silencing decreased the number of blood vessels in tumor tissues (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, nonparametric one-way ANOVA). The present in vivo study confirmed that silencing of HIF-1α or Notch1 inhibited the growth of CRC tumors and blood vessel formation within the tumors and collectively established that HIF-1α/Notch1 could be potent targets for the inhibition of angiogenesis in CRC (F. Glaus Garzon et al., 2020) ; (Cao et al., 2009).

Discussion

Colorectal cancer (CRC) is a complex disease caused through the accumulation of genetic and epigenetic aberrations such as mutations of tumor suppressor genes and oncogenes. A common genetic alteration in CRC is inactivation of the adenomatous polyposis coli gene, which increases β-catenin-mediated transcriptional activity. A recent large-scale genomic study indicated that alterations in the Notch pathway were the second most frequent after the WNT pathway. However, the role of Notch in CRC remains controversial: many studies have demonstrated that loss of Notch pathway components predisposes the colonic epithelium to hyperproliferation and polyp development, while others proposed that Notch activation promotes CRC development. Despite this controversy, it is well known that dysregulation of the Notch signaling pathway alters cell fate decision in CRC. However, the downstream target genes of Notch signaling and the key transcriptional regulators that contribute to CRC formation remain unknown.

Hypoxia-inducible factor-1α (HIF-1α) is the main regulator of the cellular response to low oxygen levels and is involved in various types of human cancer. HIF-1α drives tumor progression by promoting tumor growth, survival, angiogenesis, and metastasis. The development of anti-angiogenesis therapies targeting vascular endothelial growth factor (VEGF) has been limited due to acquired resistance and the plateau of clinical responses. Recent studies demonstrated that HIF-1α in turn regulates DLL4 expression through a HIF-1α binding site in the DLL4 promoter. As a consequence, activation of DLL4-Notch signaling inhibits angiogenesis and enhances tumor growth (F. Glaus Garzon et al., 2020). Furthermore, a recent study indicated that HIF-1α promotes malignancies in triple-negative breast cancer by activating the Notch signaling pathway. Thus, it is hypothesized that HIF-1α upregulates JAG1 expression through direct binding to hypoxia response elements (HRE) in the JAG1 promoter and hence activates Notch signaling in CRC.

Interpretation of Results

To better understand the role of HIF-1α and Notch1 in the modulation of angiogenesis in colorectal cancer, the following results were interpreted. The results of the study with respect to mRNA levels after HIF-1α and Notch1 modulation are first analyzed below. Then, the results with respect to protein levels after modulation of HIF-1α and Notch1 are analyzed. Finally, the results with respect to the tumor growth after modulation of HIF-1α and Notch1 are analyzed. For mRNA levels, the aberrant overexpression of HIF-1α in the DLD-1 and HCT-116 colon cancer cell lines was established. HIF-1α overexpression has been detected in 38 of 59 (64.4%) CRC tissues after HIF-1α knock-down and compared with that in paired adjacent tissues. No differences in HIF-1α overexpression have been found associated with age, gender, clinical stages, or histological grades. HIF-1α was shown to be inversely related to the immune microenvironmental marker CD3, and HIF-1α overexpression predicted unfavorable prognosis in CRC. Notch1 was demonstrated to be a downstream target gene of HIF-1α, which was transcriptionally activated by HIF-1α. For the protein levels, the HIF-1α/Notch1 signaling pathway was subsequently investigated and showed that Notch1 expression was significantly upregulated in the DLD-1 and HCT-116 cells after HIF-1α overexpression or hypoxia

treatment. For protein level modulation using DAPT, it was shown that Notch1 knock-down, the knock-down of its downstream targets Hes1 and Hey1, and DAPT treatment significantly inhibited HIF-1α-mediated VE-cadherin upregulation. DAPT pre-treatment reduced the compound effects of HIF-1α overexpression, hypoxia treatment, and VEGF on VEFG, Notch1, HEY1, and HES1. For tumor growth after HIF-1α modulation, the effects of HIF-1α or Notch1 modulation were evaluated. HIF-1α knock-down significantly inhibited the growth of DLD1 xenograft tumors, and most of the tumors had not formed after 35 days. HIF-1α knock-down also significantly reduced the blood vessel number and the MVD in DLD1 tumors showed smaller-sized vessels. These data showed that HIF-1α directly regulated Notch1 expression which further upregulated VE-cadherin and activated the Notch1 signaling pathway, promoting CRC cell-induced angiogenesis in a paracrine manner.

Comparison with Previous Studies

The study of colorectal cancer (CRC) has drawn much scientific attention in recent years. While the mechanisms of development and progression of many cancers have been described, there are still many aspects unknown about colon cancer. There are several studies on the roles of hypoxia-inducible factor-1α (HIF-1α) in CRC (Cao et al., 2009). The results demonstrate significantly decreased NOTCH1 and HIF-1α in most colorectal cancer tissues and cells by quantitative reverse transcription-PCR and western blotting analysis. Compared with HIF-1α-NOTCH1 groups, vascular endothelial growth factor and CD31 expression were upregulated, and ephrin-B2 was downregulated in the tissues and cells transfected with shHIF-1α or shNotch1. In comparison with the roles of HIF-1α and Notch1 in this cancer, the cross-modulating roles of HIF-1α and Notch1 were investigated, which had not been reported to date (Ahmed & Ilias, 2024). The results add new knowledge to the field of HIF-1α and related signal involved in cancer, vascular developmental capacity, and targeting therapy. Angiogenesis is a vital process during embryonic development. In the adult stage, activation is usually limited to the female reproductive cycle and wound healing. Enhancing angiogenesis may support the progression of some solid tumors. The interactions between hypoxia-inducible factor-1α (HIF-1α) and Notch1 signaling in terms of their transcriptional regulation and function modulation have not been thoroughly investigated and reported in cancer to date. The detailed mechanism studies of Notch1 as a key modulator of angiogenesis have rarely been reported regarding tumor growth. Importantly, recent studies have shown that HIF-1α is involved in Notch1 activation via an unknown molecular mechanism. It is expected that the findings will help advance the knowledge of angiogenesis and tumor development.

Conclusion

A fundamental hallmark of malignant tumors is angiogenesis, the process by which new blood vessels sprout from pre-existing ones. This process is mainly regulated by hypoxia, a characteristic property of solid tumors induced by poor blood supply. To survive and continue to expand, tumors need to stimulate angiogenesis, a phenomenon first proposed by Judah Folkman over 30 years ago. Tumor-induced angiogenesis has been regarded as a potential target for cancer therapy, and agents that inhibit angiogenesis have emerged as a new class of anticancer drugs (Cao et al., 2009).

Clinical Implications

Colorectal cancer (CRC) is the third common malignancy and the second leading cause of cancer-related death globally (Cao et al., 2009). CRC patients with distant metastasis often have a poor prognosis due to increases in tumor diameter, depth of infiltration, number of lymph nodes metastasis, and vascular and perineural invasion. The risk of distant metastasis is associated with angiogenesis, which is a critical step in cancer progression (F. Glaus Garzon et al., 2020). Hypoxia-inducible factor-1α (HIF-1α)/Notch1 is upregulated, which is able to activate to promote angiogenesis in CRC, and inhibition of HIF-1α/Notch1 causes significant inactivation of angiogenesis-related factors (Notch1, Notch3, Jagged2, Jagged1, Hes1, and VEGF). HIF-1α/Notch1-induced angiogenesis is also mediated by MVD in CRC, which is a clinical parameter of poor survival in CRC patients. The importance of this study is that it provides powerful evidence of therapeutic effects against CRC progression via the inhibition of HIF-1α/Notch1-induced angiogenesis.

CRC has been the subject of intensive research for decades. Multiple regulatory networks including oncogenes and tumor suppressor genes are involved in uncontrollable growth, aberrant cell cycle progression, invasion, metastasis, and resistance against apoptosis as well as chemotherapy and radiotherapy in CRC. Although these discoveries, there is still a lack of effective therapeutic strategies. An increased understanding of the promotion of CRC by hypoxia-induced angiogenesis is of great significance in finding new powerful therapeutic strategies. HIF-1 is a transcriptional regulator of hypoxia-inducible isolate, which is composed of a regulatory subunit (HIF-1α) and a constitutively expressed subunit (HIF-1β). Notch signaling is crucial for vascular morphogenesis and there are multiple cross talks between Notch and other signaling pathways. There is noble cross talk between HIF-1 and Notch signaling pathways. HIF-1α is involved in the maintenance and regulation of the mature vascular phenotype and inhibition of HIF-1 diminishes ACL progression in an orthotopic CRC model. The binding of ligand Jagged1 to Notch1 expressed by endothelial cell is an important Notch activation step. HIF-1 pathway upregulates Jagged1 through a regulatory circuit involving NF-kB, which is associated with inflammation and injury. However, HIF-1a/Notch1 has not been widely studied in CRC, particularly their roles in angiogenesis.

Potential Therapeutic Strategies

Despite recent advances in treatment options targeting growth factor receptors in colorectal cancer (CRC), the prognosis of patients with unresectable metastases remains poor, indicating an urgent need to search for novel therapeutic approaches. Angiogenesis is critical for tumor growth and metastasis. Among the various proteins that regulate angiogenesis, vascular endothelial growth factor (VEGF) is the most potent and specific mitogen for vascular endothelial cells. As an important proangiogenic growth factor, VEGF is upregulated by many different tumor-related pathways, such as hypoxia-inducible factor-1α (HIF-1α) (Cao et al., 2009). It has been previously shown that HIF-1α is crucial for CRC tumorigenesis. Ectopic expression of HIF-1α promoted tumor growth, which was partly due to increased levels of VEGF, glucose transporter 1 (GLUT-1), and other hypoxia-mediated genes. Blocking HIF-1α activity increased apoptosis and decreased proliferation, suggesting that targeting HIF-1α might be a promising therapeutic approach for CRC.

Despite not being a hypoxia-inducible gene, Notch1 is involved in the proangiogenic activity of HIF-1α as it regulates not only KDR/VEGFR2, the main receptor for VEGF, but also the expression of another two proangiogenic factors, IL8 and Ang1 (Zhou et al., 2020). Notch1 knockdown dramatically inhibited the CRC cell-induced angiogenesis and tube formation in cultured endothelial cells. Importantly, it was found that inhibition of endogenous HIF-1α activity in CRC cells expressing a dominant-negative HIF-1α mutant, interference of Notch1 expression in CRC cells using shRNA or pharmacological blockade of Notch signaling with DAPT significantly decreased VEGF levels and inhibited angiogenesis. These results collectively suggest that a HIF-1α/Notch1 signaling pathway activated by HIF-1α is critical for angiogenesis. Thus, combined targeting of HIF-1α and Notch may hold great promise as an effective therapeutics for CRC and possibly be of more general relevance for the treatment of cancer malignancies.

Future Research Directions

New research directions based on the findings of the current study and the context provided in the introduction are discussed. Hypoxia-Inducible Factor-1α is a key transcription factor that acts as a pivotal regulator of cellular and systemic homeostasis in response to low oxygen levels. Dysregulated expression and activity of HIFs contribute to human diseases, including cancer and cardiovascular disorders (F. Glaus Garzon et al., 2020). Inhibition of HIF has been suggested as a potential therapeutic strategy in cancer, but no HIF-targeted agent has yet been applied clinically.

The results from the current study indicated that HIF-1α activation is involved in CRC angiogenesis and that HIF-1α-mediated Notch1 expression is required for CRC cell-secreted VEGF-A165 induction. Nevertheless, the effect of Notch1-mediated inactivation of HIF-1α on possible feedback regulation of HIF-1α is still unclear and warrants further research. Current knowledge gaps on the direct roles and underlying molecular mechanisms of Notch1 in HIF-1α posttranscriptional regulation in CRC are reasonable research opportunities. Healthy quiescent vasculature relies on vascular endothelial growth factor (VEGF) to promote growth and integrity via stimulation of signalling through the VEGF receptor (VEGFR)2. High levels of VEGF activity are frequently associated with various cancers, leading to rapid proliferation and altered function of endothelial cells (Cao et al., 2009). By contrast, in normal tissues, hypoxia normally stimulates HIF-dependent increases in production of VEGF-A165 isoform, cadherins, and other direct target genes that promote angiogenesis, vascular growth, and maturation.

Conflict of Interest

No conflicts of interest were declared by the authors.

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Ethics Statement

Approved by local committee.

Authors' contributions

All authors shared in the conception design and interpretation of data, drafting of the manuscript critical revision of the case study for intellectual content, and final approval of the version to be published. All authors read and approved the final manuscript.

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