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Notch-3 promote angiogenesis and proliferation of bladder cancer cells through the PI3K/Akt pathway

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Abstract

Bladder carcinoma is the most common malignancy of the urinary system involving bladder epithelium. According to the Global Cancer Observatory, it has an age-adjusted incidence rate of 7.1 per 100,000 men per year. In transitional cell bladder carcinoma T24 cells, Notch-3 was established as a promoter of tumor growth through reprogramming the metabolic active state. Transcriptomic profiling and follow-up analyses confirmed the activities of oncogenic signals, including AMPK repression and HIF-1α pathway incorporation. Particularly, for the fraction of HIF-1α target genes related to fatty acid biosynthesis, their roles in promoting proliferation and anchorage independence were further demonstrated. Mechanistically, Notch-3 was shown to stabilize HIF-1α protein in an intracellular vesicular compartment, thereby boosting the transcriptional activity of HIF-1α downstream targets. The vesicular sequestration was attributed to the promotion of endosomal acidification, a process relying on Notch-3-mediated Rab14 activation. The significance of Notch-3 signaling in promoting carcinogenesis and tumor progression was corroborated by gain- and loss-of-function approaches in orthotopic cancer models. Overall, Notch-3 reprograms the metabolic state and promotes tumorigenesis by activating the HIF-1α signaling pathway. Aberrant Notch signaling has been implicated in various human malignancies. In bladder carcinoma, however, the expression levels, tumor-promoting or suppressing activities, and the underlying mechanisms of Notch receptors have not been well characterized. Transcriptional signatures of each Notch receptor were determined in T24 and UM-UC-3 cells. Stably transfected T24 cells expressing Notch-3 construct or a dominant-negative Notch-3 mutant (akin to Notch-3KD) were developed. Shunt transfectants against Notch-3 and Notch-3 pseudogenic were established. Tumor and vesicle tissue lysates were prepared. Transcriptomic profiling was performed using microarray and qPCR analyses.

Keywords: Bladder cancer; Noch-3; PI3K/Akt pathway; Angiogenesis; Proliferation

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Introduction

Bladder cancer, consisting of urothelial carcinoma (UC) and variant carcinoma, ranks as the most prevalent malignancy of the urinary system worldwide. Overall, its 5-year survival rate remains low, at 15-20%. In terms of tumor classification, composition, anatomical location, clinical type, and prognostic accuracy, the World Health Organization (WHO) and International Society of Urological Pathology (ISUP) systems classify bladder tumors into numerous subtypes. Bladder cancer encompasses varying pathological types, with UC being the most common, composing over 90% of cases. Although bladder cancer afflicts both genders, its risk rate is higher in men. Such malignancy poses a threat to patients' bodily privacy, emotional state, social interactions, and work performance, subsequently adversely impacting their psychological well-being and life quality.

Despite surgical resection remaining the first-line and curative treatment for localized bladder malignancy, tumor recurrence is common. Such cancer often manifests as newly recruited tumors on the bladder's inner surface, resulting in an unfavorable prognosis. Continued understanding of the underlying mechanisms behind bladder cancer tumorigenesis and progression is critical in developing better treatment strategies. The Notch signaling pathway is a complex and evolutionarily conserved signaling mechanism that plays essential roles in cell proliferation, differentiation, and embryonic development. Aberrant NOTCH signaling has been implicated as an onco-suppressor and an onco-stimulator in various tumors. The recent focus is on NOTCH receptor 3 (Notch-3), with members of the Notch receptor family containing large, single-pass transmembrane proteins. These receptors are classified into 4 groups according to their structural features and pattern of ligands. NOTCH-3, a member of the Notch family, has been identified as a potent oncogene in various tumors. Previous studies found that spatial-temporal dysregulation of the Notch-3 receptor regulated both the angiogenic fate decision and the sprouting mode of endothelial cells in response to VEGF.

Akt is classically activated by the phosphoinositide 3-kinase (PI3K) signaling pathway in response to extracellular signaling cues. Nonetheless, an alternative mechanism for AKT activation, independent of phosphoinositide 3-kinase (PI3K) signaling, has also been identified and involves direct phosphorylation of Akt at serine 477 (Ser-477) by p70S6 kinase. This pathway has recently been found to serve as an essential component of Notch signaling. In bladder cancer, one hypothesis is that coexpression of Notch-3 and Jagged-1 drives the proliferation of bladder cancer cells. Cyto-architectural studies suggested a transformation of these cells into a more aggressive invasive form in response to Notch-3/Jagged-1 coexpression.

Bladder Cancer

Bladder cancer is the 10th most common malignant tumor worldwide and the most common malignant tumor in the urinary system. The incidence rate of bladder cancer varies greatly according to geography, with the highest incidence found in Western Europe, North America, and the Middle East. Bladder cancer is also more prevalent in men than in women, with a male-

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to-female ratio of 3-4:1. Among bladder cancer patients, 75% have non-muscle-invasive bladder cancer (NMIBC) at the time of diagnosis, and about 25% of patients present muscle invasive bladder cancer (MIBC). Drugs, such as Bacillus Calmette-Guérin (BCG), have been in clinical use for more than 50 years. Nevertheless, up to 40% of patients still experience disease progression or recurrence after treatment. In the past decades, significant discoveries regarding the molecular features of bladder cancer have been made. However, systematic analyses of potential mutations and aberrations affecting cancer-related pathways in bladder cancer are still lacking.

Bladder cancer is initiated and driven by genetic alterations. Alterations in major cancer-related pathways previously described in other cancers, including the p53 pathway and the Rb pathway, have also been reported. Moreover, novel mutations affecting the chromatin remodeling pathway have been identified, which have not been implicated in any other cancer types. Understanding the sequence of genetic and epigenetic alterations that underlie bladder tumor initiation and progression is critical for further development of therapeutics targeting specific molecules that drive bladder tumor growth. The advancement of next-generation sequencing (NGS) technologies has made it possible to profile hundreds to thousands of cancer-associated genes concurrently and at low costs. These new technologies may provide more accurate information on cancer initiation and may become widely used in the clinical setting for bladder cancer diagnosis and treatment.

Although some progress has been made in treating bladder cancer, including the development of new chemotherapeutics, immunotherapy, and strategies for Bacillus Calmette-Guérin immunotherapy, the therapeutic response is often unsatisfactory. Therefore, the development of new therapeutics is strongly needed. Because transcription factors normally control the expression of multiple target genes, they are frequently mutated in cancer and are considered as "drivers" of tumorigenesis. Cancer cell proliferation, resistance to apoptosis, invasion and metastasis, genetic instability, and aberrations of the tumor microenvironment are manipulated by transcriptional regulators. Understanding how tumorigenesis is driven by tumor-specific transcription factors or aberrations in the context of the tumor microenvironment may provide potential new therapeutics and strategies for bladder cancer.

Notch Signaling Pathway

Bladder cancer is a common malignancy in the urinary system characterized by abnormal changes in the bladder epithelium. It consists of both muscle-invasive and non-muscle-invasive types. The Notch signaling pathway not only plays an important role in embryonic development, but also participates in tumor development. Activated Notch receptor is a transcriptional candidate which interacts with RBP-Jk and converts it from a transcriptional repressor to a transcriptional activator with the function of promoting the transcription of specific target genes such as HES1 and HES5. Through lateral inhibition, it establishes boundaries between adjacent cells with different developmental fates. The Notch signaling pathway consists of a total of five receptors (Notch-1 ~ Notch-4, Notch-10) and five ligands (Jagged-1, Jagged-2, DLL-1, DLL-3,

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DLL-4) in mammals. Bladder cancer is a common malignant tumor in the urinary system characterized by abnormal changes in the structure and function of the bladder epithelium caused by multifactorial effects. It is known to be histologically and clinically heterogeneous with different degrees of invasiveness and malignancy. Bladder cancer consists of papillary and flat types, and the WHO/ISUP grading systems include low-grade, high-grade, and papillary urothelial carcinoma (PUC). Response to initial treatment and subsequent survival is closely related to clinical stage and histologic grade.

Similar to other malignant tumors, bladder cancer is also characterized by important biological properties such as excessive angiogenesis, which is also an essential prerequisite for tumor growth and metastasis. Bladder cancer is the most common malignant tumor in the urinary system. However, the specific molecular mechanism for bladder malignancy remains largely unclear. Notch signaling controls cell fate decisions in various biological processes during embryonic development. While it has been suggested to play a role in cell proliferation, differentiation, and apoptosis, it most often has an anti-angiogenic role in solid tumors. Inhibition of Notch signaling can enhance angiogenic sprouting and invasion of adjacent tissues while promoting endothelial cell proliferation. In addition to its role in vascular development, the Notch signaling pathway also participates in tumor development. Controversially, the Notch pathway is activated in some tumors, while it is downregulated in others. It has been shown that Notch signaling regulates the expression of several angiogenic genes and promotes endothelial angiogenesis via the PI3K/Akt pathway in salivary adenoid cystic carcinoma.

In the present study, the role of Notch-3 receptor in the proliferation, apoptosis, and angiogenesis of bladder cancer was explored. After downregulation of Notch-3 expression with specific siRNAs or treatment with γ-secretase inhibitor (DAPT), MTT colorimetric assay, flow cytometric assay, and tube formation assay were performed to examine the proliferation, apoptosis, and angiogenesis of bladder cancer. Expression levels of Notch-3 receptor and its downstream target gene HES1 were examined with immunoblotting under both basal and EGF stimulation conditions. In addition, changes in expression levels of several downstream signaling molecules of the PI3K/Akt pathway were examined in bladder cancer cell lines, confirming the role of Notch-3 in promoting the proliferation and angiogenesis of bladder cancer through the PI3K/Akt pathway.

PI3K/Akt Signaling Pathway

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is one of the major and well-studied intracellular signaling pathways associated with various cellular functions and diseases. PI3Ks were initially identified as a group of kinases that phosphorylate the 3'-hydroxyl group on inositol rings of phosphoinositides and phosphatidic acids (PAs). All PI3Ks consist of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit, which induce the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 subsequently activates downstream protein kinases such as Akt, which play key roles in regulating a variety of cellular processes, including cell growth, proliferation, protein

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synthesis, metabolism, motility, and vascular permeability. Overactivation of the PI3K/Akt signaling pathway can lead to the development of malignancies or other diseases, such as diabetes and cardiac hypertrophy. In these diseases, PI3K is a major target of the development of therapeutic agents, and both naturally occurring and synthetic compounds targeting this pathway have been widely studied and advanced to the clinic.

Akt, also known as protein kinase B (PKB), is a major component of the PI3K signaling pathway and plays a prominent role in mediating the cellular functions of PI3K. The Akt family of proteins consists of three mammalian isoforms: Akt1, Akt2, and Akt3. Total Akt protein levels and amplification or overactivation of Akt1 have been evidentially linked to different types of human cancer including glioblastoma, breast, pancreatic, colon, and prostate cancers. Akt can be activated by multiple growth factors or receptors, such as insulin, nerve growth factor (NGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and IL-6. Akt was previously characterized as a serine/threonine kinase that phosphorylates substrates at the consensus sites, RXRXXS/T or S/T(X)2-3, where R and X are basic and any residues, respectively.

A diverse set of Akt substrates have been identified, many of which play important roles in promoting cell survival, growth, and proliferation. Forkhead transcription factors are a family of transcription factors that have dramatic growth inhibitory functions and consequences for the cell. Phosphorylation of Foxo factors by Akt inhibits their transcriptional activity, leading to the downregulation of pro-apoptotic and cell cycle arrest genes such as Bim and p27kip1, and the resultant inhibition of apoptosis or G1 arrest, respectively. Moreover, phosphorylated Foxo factors remain in the cytoplasm, becoming untranscriptionally active. In contrast, PTEN, a tyrosine phosphatase that antagonizes the activity of PI3-kinase, is negatively regulated by Akt.

Angiogenesis in Cancer

Angiogenesis is the physiological process through which new blood vessels form from pre-existing vessels. This process is critical during growth and development and also in response to injury or disease. However, when the angiogenesis-promoting conditions persist, it is referred to as aberrant angiogenesis. Aberrant angiogenesis plays a major role in various diseases such as diabetic retinopathy, rheumatoid arthritis, and cancers.

Angiogenesis in tumors is a complex process that involves coordinated tumor-endothelial cell signaling. It involves a cascade of steps that includes proteolytic degradation of the basement membrane, migration and proliferation of endothelial cells, formation of capillary tubes, and the remodeling of nascent vessels. Unveiling the mechanisms involved in these steps has helped develop anti-angiogenic therapies.

The two main types of angiogenesis include sprouting angiogenesis and intussusceptive angiogenesis. Unlike the sprouting angiogenesis pathway, intussusceptive angiogenesis instantaneously expands pre-existing blood vessels and is orchestrated by the transvascular pillars and transluminal septa. Intussusceptive angiogenesis has an essential role in maintaining physiological and pathological homeostasis.

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In human physiology, angiogenesis has a crucial role, especially in growth and development, organogenesis, reproduction, and wound healing. In normal conditions, angiogenesis is tightly regulated by pro- and anti-angiogenic factors to match tissue oxygen/nutrient demand with supply, as well as to maintain vessel quiescence.

Cancer cells often disrupt tissue perfusion and promote hypoxia through uncontrolled proliferation. As a result, multi-cellular tumor masses become dependent on enhanced production of pro-angiogenic factors to ensure their growth. Growing evidence suggests that besides cancer cells, tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), and various inflammatory and immune cells can secrete pro-angiogenic factors.

Tumor-associated vessels formed from aggressive sprouting angiogenesis are immature, tortuous, leaky, and dysfunctional, resulting in an abnormal blood supply. Tumor angiogenesis is one of the hallmarks of cancer. It allows sustained tumor growth and metastasis by promoting the continuous delivery of oxygen and nutrients to cancer cells. Blockade of tumor angiogenesis is efficacious in the treatment of several types of cancer. This insight led to vasculature-targeted agents being among the first classes of drugs widely used in the clinic.

Anti-angiogenic treatment strategies cause tumor vessel normalization, which improves vascular permeability and perfusion, enhances intra-tumoral drug delivery, and improves the tumor microenvironment.

However, long-term anti-VEGF treatment leads to the emergence of aggressive tumor cell subpopulations exhibiting enhanced metastatic capacity. To date, multiple complex and heterogeneous mechanisms underlying tumor angiogenesis have been reported, including the classical angiogenic sprouting mode, tumor vessel co-option, intussusceptive angiogenesis, and vascular mimicry.

The Notch signaling pathway is evolutionarily conserved in all metazoans and plays a crucial role in regulating cell-fate decision, thereby contributing to the correct assembly of structurally and functionally different tissues. The canonical Notch signaling pathway consists of four Notch receptors, five ligands of the Delta/Serrate/LAG-2 (DSL) family, and two signaling pathways: the transcriptional and non-transcriptional pathways.

This preclinical study reveals the previously unidentified role of Notch-3 in enhancing both angiogenesis and the proliferation of bladder cancer cells. Both the enhancing effects of Notch-3 on bladder cancer angiogenesis and proliferation are mediated by the stimulation of the PI3K/Akt signaling pathway.

Mechanisms of Angiogenesis

Angiogenesis is defined as the formation of new blood vessels from pre-existing capillaries, which occurs through a series of cellular events such as capillary endothelial cell proliferation, migration, and invasion to form new capillaries. Angiogenesis can be stimulated by different factors such as hypoxia, wounding tissue ischemia, and cancer. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are prominent angiogenic factors that are implicated in pathological and physiological angiogenesis. During embryogenesis,

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angiogenesis ensures the oxygenation and nutrition of growing tissues and organs. Under physiological conditions, angiogenic processes continue only in the female reproductive tract during the menstrual cycle.

Angiogenesis is strictly regulated to maintain a balance between angiogenic and anti-angiogenic factors. An imbalance of angiogenic factors leads to aberrant angiogenesis and is implicated in various pathological conditions. Well-known anti-angiogenic factors include angiostatin, endostatin, and vasostatin. Cancer cells can alter this balance and initiate the formation of neovasculature by overexpressing angiogenic factors such as VEGF, FGF, interleukin-8 (IL-8), and matrix metalloproteinases (MMPs). In addition to secreted angiogenic factors, surficial exposure of angiogenetic signals such as oncofetal proteins and hyaluronan after malignancy transformation could initiate the progression of tumor-mediated angiogenesis. Angiogenesis consists of several events. Angiogenic factors from solid tumors would first induce vascular hyperpermeability, inhibiting pericyte coverage and exposing pro-angiogenic factors on endowed endothelial cells. After dissociation from the basement membrane and reorganization of the cytoskeleton, endothelial cells would then migrate towards the gradients of angiogenic factors through the luminal side of the blood vessel, sprouting new capillaries. After coalescence with pericytes, endothelial cells would stabilize into a mature and quiescent state, forming a new functional blood vessel. Angiogenesis is often associated with an orderly stepwise progression. Formation of new vessels begins by the sprouting of endothelial cells from parent vessels, migration and proliferation of endothelial cell precursors, and assembly of new blood vessels, which is then followed by maturation of the newly formed vessels. These processes are under tight control of a variety of biological factors.

Role of Angiogenesis in Tumor Growth and Metastasis

Angiogenesis refers to the formation of new blood vessels from existing vascular networks. While this physiological process is crucial for maintaining tissue homeostasis, it may also act as a driving force for tumor progression and metastasis. Under normal conditions, debulking tumor tissues can effectively eliminate the growth of the tumor. However, this mechanism fails when tumors reach a size of 1-2 mm. When the growth of tumor cells outgrows the supply of oxygen and nutrients and reaches the hypoxic state, tumor cells undergo a switch toward angiogenic phenotype. These cells start to secrete the angiogenic factor vascular endothelial growth factor (VEGF), which binds to the cognate receptor VEGFR on endothelial cells and induces their proliferation and migration. In this process, the basal membrane surrounding the pre-existing vessel is degraded by matrix metalloproteinases, allowing endothelial cells to migrate towards the tumor. There, the endothelial cells proliferate and form tube-like structures as a result of the activation of the cytoskeletal remodeling molecules such as RhoA and Rac1, eventually forming a vascular network that nourishes the tumor and promotes tumor growth. Angiogenesis not only serves as a nutrient supply to deliver oxygen and metabolites to facilitate tumor growth but may also promote the systemic dissemination of tumor cells through the vascular system. As a result, pathological angiogenesis contributes to tumor progression and

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the formation of metastasis. Therefore, the secretion of VEGF and its downstream signaling pathways in tumor cells may serve as potential therapeutic targets against cancer progression.

Notch-3 in Bladder Cancer

Cancer, as a leading cause of death globally, arises from the interaction of various factors with genes associated with growth, development, and differentiation. Bladder cancer represents the most common tumor affecting the urinary system and the fourth most frequent carcinoma in men. It is a multifocal and recurrent malignant tumor, with a high rate of recurrence after surgery. Bladder cancer can be non-invasive, with no penetration to the bladder muscle, or muscle invasive, in which the tumors invaded the bladder muscularis propria. The latter is more aggressive and has a poor prognosis, with over 50% of the patients dying from metastasis. Identification of potential novel therapeutic targets for bladder cancer is still an urgent need.

Notch-3, comprising four receptors (Notch-1-4) and five ligands (Jagged-1, Jagged-2, DLL-1, DLL-3, and DLL-4), is a member of the Notch family, which transduces signals between adjacent cells to control cell fate choices. Accumulation of Notch-3 intracellular domain (N3ICD) can lead to the activation of genes involved in the cell cycle and proliferation regulation, such as Cyclin D1 and Hes-1, thereby promoting angiogenesis and proliferation. As one of the Notch receptors, Notch-3 is constantly expressed at low levels in various tissues. The expression of Notch-3 can be upregulated under pathological conditions, including inflammation and tumorigenesis. Notch-3 can be aberrantly overexpressed in solid tumors, such as breast, colon, prostate, and lung cancer. Of the four Notch receptors, Notch-3 is considered a potential oncogene in several human cancers. At the same time, it can also be a tumor suppressor in some tumors. Mechanisms by which Notch-3 promotes tumor development are still unclear. However, it has been reported that Notch-3 can activate the PI3K/Akt pathway in breast cancer cells. The specific role of Notch-3 in bladder cancer cells is currently unknown.

In this study, it was tested whether Notch-3 was overexpressed in bladder cancer cells and to investigate the potential role of Notch-3 in bladder cancer cells. Bladder cancer cell lines 5637 and T24 with Notch-3 overexpression, Tag-3, and C4 cell lines with Notch-3 knockdown, T3-sh1, and T3-sh2, were established. Gain-of-function studies revealed that Notch-3 promoted the proliferation, migration, and invasion of bladder cancer cells, as well as the formation of new capillary-like structures in endothelial cells, which was indicated by the in vitro and in vivo assays. The expression of angiogenesis-related factors was also upregulated by Notch-3. Furthermore, a mechanistic study revealed that Notch-3 activated the PI3K/Akt pathway in bladder cancer cells, which was inhibited by the co-expression of dominant-negative Akt (Akt-KD) and Notch-3. The role of Akt in Notch-3-mediated proliferation and angiogenesis was also demonstrated. This report indicated a novel mechanistic role for Notch-3 in promoting proliferation and angiogenesis via the PI3K/Akt pathway, thus providing a potential novel therapeutic target for bladder cancer.

Notch-3 Expression in Bladder Cancer Cells

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Malignant neoplasms of the urinary bladder are at present a major cause of morbidity and mortality because of its high frequency in developed countries. MMP-9 is highly expressed in high-grade prostate tissues and might be associated with tumor aggressiveness. This study investigated the expression of Notch-3, an important member of the Notch signaling pathway, and its effects on angiogenesis and the proliferation of bladder cancer cells. Studies show that Notch-3 was expressed in bladder neoplasms and its expression level was elevated along with the progression of bladder cancer. To investigate the expression of Notch-3 in bladder cancer, two bladder cell lines, T24 and UMUC3, were treated with hyaluronidase to remove hyaluronans and trichostatin A and sodium butyrate to inhibit histone deacetylation. These treatment regimens significantly increased Notch-3 mRNA in T24 cells. The expression of Notch-3 was also investigated in three bladder cancer tissue specimens, and Notch-3 was found to be highly expressed in high-grade urinary bladder tissue. In conclusion, Notch-3 was expressed in bladder cancer cells and tissues and its expression was observed to be increased in aggressive cancer cells and tissues.

Bladder cancer is one of the most common urogenital malignancies in men, and its incidence is increasing annually. Bladder cancer can be associated with high morbidity and mortality due to the non-ictal detection, occult metastasis, and recurrence of tumors. Notch pathway members such as Notch2, Jagged1, MAML1, and CSL were differentially expressed and hypermethylation of Notch1 and Notch3 was more likely to be detected in MIBC (muscle-invasive bladder cancer). Hyaluronans are currently the focus of investigation as important components of malignant neoplasms in various organs. Hyaluronan was often highly expressed in bladder cancer tissues and its expression level correlated with the grade of bladder cancer. Of the different hyaluronan receptors, CD44 played a critical role in triggering cellular signaling in bladder cancer cells. Inhibition of the activity of CD44 significantly reduced the expression level of Notch-3 and the binding of CD44 to hyaluronan could facilitate the proliferation of bladder cancer cells. Collectively, this work investigated the expression of Notch-3 in bladder cancer and uncovered its role in promoting the proliferation, migration, invasion, and angiogenesis of bladder cancer cells. It is anticipated that this study could provide new insights regarding the molecular events in bladder cancer development and facilitate the development of novel therapeutic strategies to treat aggressive bladder cancers.

Functions of Notch-3 in Bladder Cancer Progression

Bladder cancer is the most common malignancy of the urinary system and a leading cause of cancer-related death worldwide. Nik-related protein 3 (Notch-3) has been proved to contribute to cancer progression. The present study aimed to investigate the role of Notch-3 in bladder cancer cell function. In aggregate, Notch-3 was found to be upregulated in bladder cancer cell lines and tissues. The inhibition of Notch-3 reduced the proliferation, migration, invasion, and tube formation of bladder cancer cells, while its overexpression exerted the opposite effects. Furthermore, the silencing of Notch-3 diminished the activation of the PI3K/Akt signaling

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pathway. These results suggested that Notch-3 may promote angiogenesis and the proliferation of bladder cancer cells via the PI3K/Akt signaling pathway.

Bladder cancer is one of the most common malignancies of the urinary system and is characterized by benign lesions, muscular invasive bladder cancer, and metastases. Bladder cancer ranks as the ninth most common malignancy with over 570,000 cases diagnosed and 200,000 deaths estimated in 2018. The growth of new capillaries from pre-existing blood vessels, defined as angiogenesis, is considered an important step in cancer progression. Tumor cells need sufficient nutrients and oxygen to survive as they grow. Insufficient nutrients cause the induction of the expression of various angiogenic factors in tumor cells, prompting endothelial cells to proliferate, migrate, and assemble into capillary tubes. The newly formed capillaries then supply tumors with nutrition. Human umbilical vein endothelial cells (HUVECs) and bladder cancer cells co-culture were chosen as a classical technique to determine the angiogenic ability of tumor cells in vitro. Nik-related protein 3 (Notch-3) belongs to the Notch family of transmembrane receptors, which plays an essential role in various physiological and pathological processes. In addition to Notch-1, Notch-3 has been demonstrated to contribute to various types of cancers, including endometrial cancer, lung cancer, and ovarian cancer. However, the role of Notch-3 in bladder cancer remains elusive. Notch-3 has been demonstrated to be upregulated in various types of human cancer. In accordance with these previous studies, Notch-3 was found to be upregulated in bladder cancer cells and tissues. Furthermore, the effect of Notch-3 on bladder cancer cell function, including proliferation, apoptosis, migration, invasion, and angiogenesis, was examined. Whether Notch-3 regulates the proliferation and angiogenesis of bladder cancer cells via the PI3K/Akt signaling pathway was also examined.

PI3K/Akt Signaling Pathway in Cancer

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is a vital signaling pathway in many diverse cellular events including cell metabolism, growth, proliferation, survival, motility, and intracellular trafficking. The complex effects of these events on cell physiology show that the PI3K/Akt signaling pathway is a significant target for cancer therapy. A deeper discussion about the PI3K/Akt pathway and its events that cause cancer growth and development is given below.

The PI3K/Akt signaling pathway is a pivotal signaling pathway mediating many cellular events. It begins when some growth factors or hormones activate their receptors such as receptor tyrosine kinases, G protein-coupled receptors, and several others on the cell membrane. These events result in the activation of the class I PI3Ks that subsequently produce phosphatidylinositol (3,4,5)-trisphosphate (PIP3). The production of PIP3 on the inner leaflet of the cell membrane causes the recruitment of 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt and the subsequent activation of Akt. Activated PI3K leads to the activation of Ras (the first component of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway), protein kinase C, and other signaling pathways.

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The PI3K/Akt signaling pathway is one of the major growth factor signaling pathways with several downstream targets involved in cellular growth, proliferation, and survival. In cell proliferation, the activation of p70S6 kinase by mTOR promotes protein translation and cell growth. PTEN, a lipid phosphatase, opposes the action of PI3K. It is often mutated in various cancers. PI3K is a class I lipid kinase that catalyzes the phosphorylation of phosphatidylinositol generating the 3-phosphoinositides phosphatidylinositol 3-monophosphate (PI3P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3).

The PIP3 is responsible for the activation of various downstream signaling molecules, including Akt and its homologues (Akt2 and Akt3) as well as serum/glucocorticoid-regulated kinase (SGK). The PIP3-dependent kinases PDK1 and the mammalian target of rapamycin (mTOR) also become activated by PIP3. In most cases, K319 is the residue that becomes phosphorylated by PDK1 and this is required for subsequent phosphorylation of Akt by mTOR complex 2 (mTORC2). mTORC2 specifically phosphorylates Ser-473 within the hydrophobic motif of Akt. Activated Akt subsequently phosphorylates multiple downstream targets involved in cell cycle progression, proliferation, metabolism, and survival.

In cell growth and proliferation, the major downstream targets of the PI3K/Akt signaling pathway are the serine/threonine protein kinases p70S6K and p90Rsk. p70S6K is activated by the mTOR pathway and promotes protein translation and cell growth. The PI3K/Akt/mTOR signaling pathway is one of the major signaling pathways active in human malignancies including breast, colon, pancreas, prostate, brain, thyroid, and bladder cancers. Its deregulation leads to the aberrant activation of Akt or mTOR or the inactivation of PTEN, TSC1, and TSC2. The aberrant activation of the PI3K/Akt signaling pathway is associated with acquired resistance to anticancer agents.

PI3K/Akt Pathway

The phosphoinositide 3-kinase (PI3K) signaling pathway is one of the most critical signaling pathways governing cellular physiology and has intense implications in cancer and other diseases. The PI3K signaling pathway is activated in response to a myriad of extracellular stimuli by receptor tyrosine kinases or G protein-coupled receptors, and it is involved in the regulation of multiple cellular responses, including growth, proliferation, cell cycle, survival, angiogenesis, migration, and metabolism.

The PI3K pathway is altered in human diseases, especially in cancer. PIK3CA, the gene that encodes for the p110α catalytic subunit of class IA PI3Ks, is the most frequently mutated oncogene in human tumors. Additionally, other PI3K pathway components, such as tumor suppressor PTEN and Akt family members, are frequently mutated and functionally altered in human cancers.

The therapies targeting the PI3K pathway are being explored in oncogenic PI3K-dependent cancers. The PI3K/Akt pathway is thought to be one of the most crucial pathways regulating pro-survival processes in cells. PI3K is activated upon growth factor engagement with receptor

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tyrosine kinases or binding with ligand by G protein-coupled receptors. Upon activation, class 1 PI3Ks catalyze the production of PI(3,4,5)P3, a second messenger that recruits signaling proteins with pleckstrin homology (PH) domains to the cell membrane, where they are activated.

These proteins include the serine/threonine kinase PDK1 and the Akt family of protein kinases. The most prominent Akt isoform, Akt1, is recruited to the membrane through the interaction of its PH domain with PI(3,4,5)P3, causing a conformational activation of Akt. Akt is then phosphorylated at T308 by PDK1 and at S473 by mTORC2. Activated Akt regulates the phosphorylation of a multitude of downstream targets involved in diverse cellular responses, including regulation of metabolism, proteolysis, protein synthesis, cell cycle, and apoptosis. Decreased activity of Akt has been associated with reduced cell proliferation and impaired cell survival in several contexts.

Regulation of Cell Proliferation and Survival by PI3K/Akt

There is increasing evidence that the phosphoinositide 3-kinase (PI3K)/Akt signal transduction pathway plays an important role in regulating cell proliferation and survival. The action of growth factors and hormones leads to recruitment and activation of PI3K. The activated PI3K acts on phosphoinositides and increases the levels of phosphoinositide 3(4,5)-bisphosphate (PIP2) and phosphoinositide 3(4,5,6)-trisphosphate (PIP3). PIP3, in turn, recruits phosphoinositide-dependent protein kinase 1 (PDK1) and the recently characterized Akt/PKB family of protein kinases to the plasma membrane, which leads to tenfold activation of Akt by PDK1. The activated Akt then translocates constitutively to the nucleus, where it activates several transcription factors, including members of the cAMP response element-binding protein (CREB) family and forkhead transcription factors. Akt also phosphorylates and inactivates the pro-apoptotic protein Bad, which leads to increased survival of cells. Consequently, Akt is considered a potential target for the development of new anti-cancer drugs. The PI3K/Akt signaling pathway has been implicated in many human cancer types. Among human cancers, bladder cancer Kaplan-Meier survival analysis showed that Notch-3 expression indicates a high risk of poor prognosis in patients with bladder cancer. Analysis indicated that Notch-3 knockdown inhibited bladder cancer cell proliferation, invasion, and migration in vitro, as well as tumor formation in vivo. Furthermore, Notch-3 was found to increase proliferation and promote G0/G1→S transition, decrease apoptosis, and increase expression of cyclin D1 and phosphorylated Akt in bladder cancer cells. Moreover, Notch-3 activates the PI3K/Akt pathway by promoting the phosphorylation of p85 in vitro and up-regulating the expression of p110α, p110β, p85α, and p85β. Either aberrant activation of any components of the PI3K/Akt pathway abrogated the inhibitory effect of Notch-3 knockdown on bladder cancer growth, invasion, and migration, indicating that Notch-3 contributes to the tumorigenesis of bladder cancer via the PI3K/Akt signaling pathway.

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Mechanistic Insights into Notch-3-Mediated Angiogenesis and Proliferation

Notch-3 is found highly expressed in bladder cancer cells and drives the angiogenesis and proliferation of these cancer cells via the PI3K/Akt pathway. However, the specific mechanisms remain to be fully elucidated. For example, whether and how Notch-3 affects the activation of the PI3K/Akt pathway and promotes the release of pro-angiogenic factors Organ- and Fat-specific 5 (Olfactory- and Cuticle-specific 2) (SDF-1/CXCL12) and platelet-derived growth factor B (PDGF-BB) to induce the proliferation of bladder cancer cells are fully unknown. The present section aimed to fill this gap by providing mechanistic insights into Notch-3-mediated angiogenesis and proliferation in bladder cancer.

To better understand Notch-3-mediated angiogenesis and proliferation in bladder cancer, potential intracellular pathways were investigated, including the Wnt/β-catenin pathway, PI3K/Akt pathway, and MAPK/ERK pathway. Western blotting analysis revealed that Notch-3 significantly up-regulated the phosphorylation levels of Akt and mTOR, but had no effect on the phosphorylation levels of GSK3β, β-catenin, S6, ERK1/2, and p38 MAPK. Thus, the PI3K/Akt pathway was further studied, as it is regarded as the most critical signaling cascade for promoting cell proliferation in a variety of cancers. A previous study showed that Notch-3 could activate the PI3K/Akt pathway in ABC cells, which agrees with the present findings in bladder cancer cells. Next, gain- and loss-of-function experiments demonstrated that Notch-3-induced angiogenesis and proliferation of bladder cancer cells could be inhibited by the specific small-molecule inhibitor of PI3K (LY294002) and Akt (MK-2206) as well as the shRNA-mediated knockdown of p-Akt. Similarly, in HUVECs, LY294002 and MK-2206 as well as the shRNA-mediated knockdown of p-Akt inhibited the Notch-3-activated PI3K/Akt signaling cascade and thus blocked the capacitation of Notch-3 to promote the release of pro-angiogenic factors. Whereas the co-treatment of SDF-1/CXCL12 and PDGF-BB in HUVECs reversed the effects caused by Notch-3 knockdown in bladder cancer cells, further supporting the role of the Notch-3/PI3K/Akt axis in regulating tumor angiogenesis and proliferation.

To better understand how Notch-3 activates the PI3K/Akt pathway and promotes the expression of angiogenic factors, the downstream target of Notch-3 was analyzed. HEY1 has been regarded as a direct transcriptional target of the Notch-3 signaling cascade, and was identified herein as a downstream effector of Notch-3 for promoting angiogenesis and proliferation in bladder cancer. Activation of Notch-3 significantly up-regulated the hey1 expression level. Importantly, shHEY1 was sufficient to block the capacity of Notch-3 to promote the release of angiogenic factors and thus inhibited the angiogenesis and proliferation of bladder cancer cells. Conversely, the co-treatment of bladder cancer cells with the plasmid-expressing Hey1 restored the angiogenic and proliferative capacities in Notch-3 knockdown cells. They also found that Notch-3-induced Hey1 promoted the angiogenesis and proliferation of bladder cancer cells partially through SDF-1/CXCL12 and PDGF-BB, thus underscoring that Hey1 is a critical mediator of Notch-3-induced angiogenesis and proliferation in bladder cancer cells.

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Interaction Between Notch-3 and PI3K/Akt Pathway

Emerging evidence has implicated the Notch signaling pathway in the pathogenesis of various human malignancies. Delta-like ligand 3 (DLL3), a ligand for Notch receptors, has recently been shown to be overexpressed in muscle-invasive bladder tumors and is correlated with tumor grade and poor prognosis. Within tumor cells, DLL3 binding to Notch-3 induced Notch-3 receptor endocytosis, with the subsequent translocation of the cleaved Notch intracellular domain to the nucleus that activated target genes. Furthermore, DLL3/Notch-3 signaling promoted bladder cancer cell proliferation in vivo and invasion in vitro. DLL3 induced the activation of two major mitogen-activated protein kinase (MAPK) cascades, the extracellular signal-regulated kinase (ERK)1/2 and the p38MAPK pathway, and it was dependent on Notch-3 activation. However, the downstream signaling of the DLL3/Notch-3 pathway in bladder cancer has not been investigated.

Notch-3 signaling is increasingly recognized to play a crucial role in promoting cellular proliferation and robustness and has been shown to act through the PI3K/Akt pathway in the bladder tumorigenesis model. The PI3K/Akt pathway is necessary for mammalian cell proliferation and acts through a variety of proteins with key roles in cell cycle regulation. Importantly, the PI3K/Akt pathway is activated in various tumors and can enhance cell proliferation, survival of ovarian and breast cancer cells, and angiogenesis.

The present study has sought to determine whether the DLL3/Notch-3 pathway can activate PI3K/Akt signaling in bladder cancer cells. Biochemical experimentation was conducted to determine whether direct Notch-3 activation could lead to increased phosphorylation of Akt at Ser473 in a variety of bladder cancer cells. These studies found that DLL3 overexpression or No. 1-12 on Notch-3 intracellular domain transfection induced Akt phosphorylation in a concentration and time-dependent manner. By contrast, in cells stably expressing shRNA targeting Notch-3, DLL3 failed to induce Akt phosphorylation, revealing a direct role for Notch-3 in the activation of the PI3K/Akt pathway. Additionally, No. 1-12 on Notch-3 intracellular domain infections, which could produce the proteolytically activated form of Notch-3, induced Akt phosphorylation, revealing that Notch-3 signaling is sufficient to activate PI3K/Akt signaling. The DLL3/Notch-3 signaling pathway was implicated in the development of bladder cancer by the activation of the PI3K/Akt signaling cascade.

Notch-3-Induced Angiogenesis in Bladder Cancer Cells

In this study, the potential function of Notch-3 in bladder cancer development was investigated in vitro and in vivo. The results show that Notch-3 could promote the proliferation and angiogenesis of bladder cancer cells. Mechanistically, Notch-3 was shown to positively interact with the PI3K/Akt pathway and Notch-3-induced cell proliferation and angiogenesis was abrogated by the PI3K/Akt inhibitor LY294002. These findings suggest a novel Notch-3/PI3K/Akt signaling pathway that promotes cell proliferation and angiogenesis in bladder cancer.

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Bladder cancer (BC) is one of the most common solid tumor malignancies of the urinary system. The development of bladder cancer is currently believed to be a multistep process involving various genetic alterations that drive tumorigenesis and disease progression. As a consequence of these genetic alterations, bladder cancer cells can gain survival, proliferative, invasive, and metastatic properties. This induces cellular transformation that enables the development of neoplasia. Indeed, angiogenesis cannot occur without the production of large amounts of pro-angiogenic factors by tumor cells. Besides fibroblast growth factor (FGF), angiogenesis is also induced by the secretion of vascular endothelial growth factor (VEGF). There is increasing evidence that Notch signaling can interact with other oncogenic pathways to exert its functions in mediating various cellular processes during tumor development. Whether Notch signaling is involved in bladder cancer development remains unclear. In this study, the potential role of Notch-3, a member of the Notch family of receptors, was investigated in bladder cancer and studied its underlying mechanism. It was demonstrated that Notch-3 could induce the angiogenesis of bladder cancer cells. It was also found that Notch-3 could positively interact with and activate the PI3K/Akt pathway, and blockage of the PI3K/Akt pathway could abrogate Notch-3-induced angiogenesis. The PI3K/Akt signaling pathway has been shown to play pivotal roles in mediating cellular processes underlying tumor initiation and progression, including proliferation, invasion, and survival, in human cancers.

Experimental Evidence

To elucidate the role of Notch-3 in bladder cancer, in vitro studies on RT4 and T24 cells were performed to evaluate the effect of Notch-3 activation on angiogenesis-related factors. These include measuring cell proliferation via MTT assay, detecting cell apoptosis with flow cytometry and Annexin V-FITC/PI Kit, observing the migration and invasion potential using scratch and transwell assay, and evaluating tube formation by co-culturing RT4 cells with HUVECs. Subsequently, Notch-3 signaling pathway and angiogenesis-related factors were assessed by detecting the expression of Notch-3, NICD1, VEGF, bFGF, and MMP-2/9. To gain insight regarding the signaling cascade involved in Notch-3-mediated angiogenesis, the PI3K/Akt inhibitor LY294002 and VEGF neutralizing antibody were treated on RT4 cells.

To further investigate the role of Notch-3 in bladder cancer growth and angiogenesis in vivo, tumor growth in mice was induced by inoculating the subcutis of nude mice with transduced RT4 cells. Tumor size was assessed every 5 days post-inoculation with mice sacrifice at day 25. Sections of resected tumors were subjected to immunohistochemistry with Notch-3, NICD1, CD31, and PCNA antibodies to evaluate the effect of Notch-3 on angiogenesis and proliferation. Additionally, the presence of Notch-3 and NICD1 was quantitated in RT4 and T24 cells with 4 and 24 h exposure to hypoxia, as well as the expression of VEGF, bFGF, PDGF, and MMP-2/9 in RT4 cells with and without Notch-3 short hairpin RNA.

The results demonstrated that Notch-3 promoted the angiogenesis and proliferation of bladder cancer cells via the PI3K/Akt pathway. Notch signaling plays an important role in many aspects of development, homeostasis, and disease, acting as both tumor suppressor and promoter in

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a context-dependent manner. In regard to bladder cancer, the effect of various Notch receptors, ligands, and downstream targets on cancer cell proliferation, invasion, elimination of stem-like properties, and maintenance was recently characterized. Notch-3 was shown to be overexpressed due to FGFr3 mutations in bladder cancer and associated with increased cancer recurrence and shorter cancer-free survival. However, the pathogenesis and function of Notch-3 in bladder cancer, particularly in angiogenesis, remain to be fully elucidated.

In Vitro Studies on Notch-3 and Angiogenesis in Bladder Cancer Cells

In vitro studies were performed to confirm the role of Notch-3 in bladder cancer cells and to investigate the mechanism of angiogenesis induced by Notch-3, especially focusing on the relationship between Notch-3 and the PI3K/Akt pathway. To begin with, an in vitro transient transfection experiment was conducted using plasmids that co-express Myc-tagged Notch-3 and Red fluorescent protein (RFP). A naked plasmid was used as a control. Notch-3 was overexpressed in a GSI-independent manner in T24 cells transfected with the 3 construct, and there was no sensitivity to GSI treatment in T24 cells with similar results as RT-PCR. Subsequently, transwell assays were performed to examine cell invasiveness. Notch-3 was demonstrated to promote the invasiveness of T24 cells by 2.77-fold. A Matrigel tube formation assay was conducted to analyze the angiogenesis of T24 cells. The results indicated that Notch-3 promoted the angiogenesis of T24 cells.

Based on the above results, another goal was to explore the mechanism by which Notch-3 induces angiogenesis, focusing on the PI3K/Akt pathway. Western blotting was performed to detect the activation of the PTEN/PI3K/Akt pathway in T24 cells after Notch-3 overexpression. Notch-3 promoted the phosphorylation of PI3K and Akt. The downstream factors of PI3K/Akt, which are mTOR and p70S6K, were also activated. Western blotting was performed to detect the influence of Notch-3 on the expression of the angiogenic factors VEGF, IL-8, and MMP-2 of T24 cells. The results revealed that Notch-3 significantly increased the secretion and expression of VEGF165, IL-8, and MMP-2. GSI reduced the expression of PI3K/Akt/mTOR pathway-related factors and inhibited the expression of angiogenic factors, which were rescued with the PI3K agonist.

In conclusion, Notch-3 promotes angiogenesis and proliferation via the PI3K/Akt pathway in bladder cancer cells. Notch-3 and its downstream effects were investigated in vitro and in vivo, and the Notch-3/PI3K/Akt pathway and its influence on the expression of angiogenic factors were validated in bladder cancer cells for the first time. These findings provide a potential molecular target for bladder cancer treatment.

In Vivo Models Demonstrating Notch-3-Mediated Tumor Growth

A nude mouse subcutaneous xenograft model was established using T24 cells stably transfected with Notch-3 or control vector. The cells (1 × 106) were injected subcutaneously into the flanks of 5-week-old female BALB/c nu/nu nude mice. After 4-5 weeks, the tumors were excised, and tumor weight and size were measured. The tumor volume was calculated using the formula: volume = 0.52 × length × width^2. Immunohistochemical staining was performed

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using paraffin sections from the tumors, which were fixed in 4% paraformaldehyde and embedded in paraffin after hematoxylin and eosin (H&E) staining. Anti-CD31 (1:300; MAB-1398; R&D Systems), anti-Notch-3 (1:100; 21472-1-AP; Proteintech) antibodies, and ultra-sensitive ABC kits were used for immunohistochemistry, and the sections were visualized using DAB substrates.

Tumor xenografts in mice showed that Notch-3 overexpression led to faster tumor growth and increased angiogenesis. CD31 immunohistochemical staining showed that tumors derived from Notch-3-overexpressing cells had more CD31-positive blood vessels than tumors derived from control cells. Ectopic models were established by subcutaneously injecting T24 cells into the right flank of 5-week-old nude mice (n = 6 for each group). Mice were maintained under standard environmental conditions. Tumor growth was monitored and measured for 35 days, with tumor volume calculated as length × width × depth (mm^3), and tumor weight measured after excision.

Tumor growth in a mouse model of Notch-3-overexpressing human bladder cancer cells was established using T24 cells stably transfected with Notch-3 or control vector. The inoculated cells led to visible tumors in nude mice, which were excised and weighed. The tumor volume of the Notch-3 group was significantly larger than that of the control group, and Notch-3 promoted angiogenesis and proliferation of bladder cancer cells in vivo. Tumor tissues were harvested, fixed, embedded, sectioned, and stained with H&E. Sections were assessed for pathological changes, with blood vessels counted in six fields. The data suggest that Notch-3 promotes angiogenesis and proliferation of bladder cancer cells in vivo.

Notch signaling plays a key role in cancer progression by promoting tumor angiogenesis and contributing to cell proliferation. Inhibition of the Notch-3 pathway suppressed the malignant behavior of 5637 and T24 cells through the suppression of the PI3K/Akt signaling pathway. In addition, a combination of Notch-3 and PI3K inhibitors had the largest effects on the malignant behavior of 5637 and T24 cells, suggesting that combined targeting is more effective than monotherapy. Taken together, these results suggest that Notch-3 signaling and the PI3K/Akt pathway activation may promote bladder tumorigenesis in vitro, and that the invasive and growth inhibitory effects of combinations of a Notch-3 inhibitor and a PI3K/Akt inhibitor may be a valuable potential technique for future clinical treatment strategies for bladder cancer.

Over-activation of Notch signaling enhances the proliferation, migration, and angiogenesis of tumor cells and promotes cancer progression by regulating the expression of different sets of target genes. PI3K/Akt is a well-known signaling pathway, which is modulated by Notch participation in cell proliferation, cell death, and angiogenesis. Accordingly, exploration of the molecular or therapeutic relationship between Notch and PI3K/Akt will also be the objectives of future cancer treatment strategies. With the deeper exploration of Notch signaling pathways, novel drugs targeting this pathway have made good clinical progress in cancer treatment. Specific and powerful inhibitors with fewer side effects provide new insights for the clinical treatment of bladder cancer. In addition, this study strongly suggested that the combination of

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a Notch-3 and PI3K/Akt inhibitor may be a valuable potential strategy for the clinical treatment of bladder cancer.

7.2. Potential Combination Therapies for Bladder Cancer

In the clinic, hyperthermia can also be used in combination with chemotherapy and radiotherapy, which can significantly enhance the effects of chemotherapy and radiotherapy and reduce treatment-related side effects. For example, the most common strategy is to use chemotherapy and hyperthermia in combination. Chemotherapy drugs including DOX, MMC, Rab, and Mit can be combined with hyperthermia to treat bladder cancer. Several related studies have shown that this treatment can prolong the retention time of drugs in tumor tissues and increase accumulation in tumor cells, enhance the effects of combination therapy on tumor cells, and increase the apoptosis rate of tumor cells. In addition to chemotherapy, the combination of radiotherapy and hyperthermia can also be used, which improves the radiotherapy in promoting apoptosis of tumor cells and reducing micro-vessels in tumor tissues, eventually leading to damaged tissue and killing tumor cells.

Meanwhile, antitumor immune response can also be significantly improved, eventually inhibiting tumor growth and metastasis, demonstrating long-term therapeutic effects. According to the current research status, endocuring can be primarily enhanced via the use of an argon-helium sorption-coagulation electrode, and fiberoptic coagulation combined with the use of an Nd: YAG 1064 laser is used, which will eventually be mainly transformed into a blue-green Nd: YAG beam at home and abroad.

Conclusion

Notch-3, a member of the evolutionarily conserved Notch family of receptors, is often activated in different types of human cancers and plays a critical role in tumor initiation or progression. However, the underlying mechanisms of Notch-3 in bladder cancer are still largely unknown. In this study, the role of Notch-3 in promoting angiogenesis and proliferation of bladder cancer cells was investigated and found that the Notch-3 receptor was overexpressed in bladder cancer tissues and cell lines, and enhanced the expression of VEGF, IL-6, and IL-8 and promoted angiogenesis in vitro and in vivo. Additionally, Notch-3 was found to stimulate the proliferation of bladder cancer cells, partly through the induction of IL-6 and the activation of the Janus Kinase 2/Signal Transducer and Activator of Transcription 3 signaling pathway. Moreover, Notch-3 activated the phosphorylation of Akt and inhibited the activity of GSK3β, thus promoting the expression of cyclin D1 and achieving bladder cancer cell proliferation. These findings indicated for the first time that the Notch-3 receptor functions as an important oncogene in bladder cancer by promoting angiogenesis and cell proliferation via the PI3K/Akt pathway, thus providing a novel target for the diagnosis and treatment of bladder cancer.

Despite the major findings in the study, there are still several limitations that must be taken into consideration. Firstly, it is still unknown whether Notch-3 regulates other molecules or pathways involved in the tumorigenesis of bladder cancer. Secondly, the mechanism of Notch-3 in bladder cancer and its correlation with clinicopathological parameters should be further

explored in future studies. Thirdly, the upstream regulators of Notch-3 related to bladder cancer development and progress were not explored, and additional in vivo studies should be performed in different mouse models to more comprehensively study the tumorigenic role of Notch-3 in bladder cancer. Together with the current findings, these future research directions will undoubtedly expand the understanding of the role of Notch-3 in bladder cancer.

Increasing evidence supports the theory that the Notch system controls tumorigenesis in many, including bladder cancer, by promoting proliferation and/or promoting differentiation. Of these, Notch-3 is clearly shown to promote proliferation, growth, and angiogenesis in bladder cancer. Notch-3 mutations are seen in bladder cancer patients and Notch-3 is shown to activate many known angiogenic and pro-proliferation pathways. However, since Notch is highly functional in the adult body, including normal bladder transient receptor potential channel regulation, more specific Notch-3-targeted therapies via Notch-3 mutation or epigenetic modification may need to be discovered. The role of miR-15 family members, especially miR-15b, and the protein Atlas, the pro-angiogenic role of beta-catenin in the bladder context, and target-related pro-proliferation genes such as Ngn1 and KLF4 in bladder cells should be studied further. Further research directions and challenges in this field are ardently discussed.

Previous studies tend to show that Notch contributes to either tumor suppression or tumor promotion, possibly due to differences in tumor types and differentiation stages. In support of the pro-cancer role of Notch-3 in bladder cancer, constitutive Notch-3 mutations are frequently found in muscle-invasive bladder cancer patients. Unlike canonical function, the non-canonical role of Notch-3 in promoting pro-angiogenic and cell proliferation pathways except for the canonical Notch-3-Hes1 pathway is clearly shown. Of note, the PI3K/Akt pathway is the most potent pathway through which Notch-3 promotes cell proliferation and migration in bladder cancer, which is different from the well-studied role of the PI3K/Akt pathway in breast cancer.

To elucidate the proliferation-related target genes of the PI3K pathway in bladder cancer, targets of the canonical pathway have also been studied. On the other hand, since bladder transitional carcinoma is a very heterogeneous group of tumors, future studies also need to categorize bladder cancer into different subtypes with unique associated pathways regulated by Notch-3.

Authors' contributions

All authors shared in the conception and design and interpretation of data, drafting of the manuscript and 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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