Nestin: A novel angiogenesis marker and possible target for tumor angiogenesis

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Abstract

Abnormal vasculature, termed tumor vessels, is a hallmark of solid tumors. The degree of angiogenesis is associated with tumor aggressiveness and clinical outcome. Therefore, exact quantification of tumor vessels is useful to evaluate prognosis. Furthermore, selective detection of newly formed tumor vessels within cancer tissues using specific markers raises the possibility of molecular targeted therapy *via* the inhibition of tumor angiogenesis. Nestin, an intermediate filament protein, is reportedly expressed in repair processes, various neoplasms, and proliferating vascular endothelial cells. Nestin expression is detected in endothelial cells of embryonic capillaries, capillaries of the corpus luteum, which replenishes itself by angiogenesis, and proliferating endothelial progenitor cells, but not in mature endothelial cells. Therefore, expression of nestin is relatively limited to proliferating vascular endothelial cells and endothelial progenitor cells. Nestin expression is also reported in blood vessels within glioblastoma, prostate

cancer, colorectal cancer, and pancreatic cancer, and its expression is more specific for newly formed blood vessels than other endothelial cell markers. Nestin-positive blood vessels form smaller vessels with high proliferation activity in tumors. Knockdown of nestin in vascular endothelial cells suppresses endothelial cell growth and tumor formation ability of pancreatic cancers *in vivo*. Using nestin to more accurately evaluate microvessel density in cancer specimens may be a novel prognostic indicator. Furthermore, nestin-targeted therapy may suppress tumor proliferation *via* inhibition of angiogenesis in numerous malignancies, including pancreatic cancer. In this review article, we focus on nestin as a novel angiogenesis marker and possible therapeutic target *via* inhibition of tumor angiogenesis.

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Key words: Nestin; Angiogenesis; Molecular targeting therapy; Gastrointestinal cancer; Pancreatic cancer; Microvessel density; CD34; CD31; Factor VIII

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INTRODUCTION

A hallmark of solid tumors is abnormal vasculature, termed tumor vessels, which is adjacent to the tumor itself. Greater number of tumor vessels increases the risk that tumor cells will enter systemic circulation. The basement membranes of newly formed tumor vessels are highly permeable, enabling tumor cells to penetrate them more easily than mature vessels^[1]. Furthermore, tumor angiogenesis enhances the supply of oxygen and nutrients to solid tumor cells. As a result, the tumor grows more rapidly and easily when tumor vessels are formed

in close proximity. After a tumor has attained a size of 1-2 mm, the induction of new blood vessel formation is required for further growth and expansion^[2]. In fact, the degree of tumor angiogenesis reportedly correlates with clinical outcome, and angiogenic properties correlate with tumor aggressiveness in gastrointestinal (GI) cancers including colorectal cancers^[3], gastric cancers^[4], esophageal cancers^[5], and pancreatic cancers^[6,7]. These findings suggest that exact quantification of tumor vessels is useful to evaluate prognosis. Furthermore, selective detection of newly formed tumor vessels within cancer tissues using specific markers raises the possibility of molecular targeted therapy *via* the inhibition of tumor angiogenesis. Microvessel density (MVD), measured immunohistochemically in tumors, is often reported to correlate with prognosis in various malignancies^[8,9], including GI cancers^[10-14]. However, the clinical significance of MVD is controversial; for example, pancreatic cancer is characterized by hypovascularization^[15,16]. For MVD analysis, CD34, CD31, and factor VIII are commonly used as endothelial cell markers in tumor vessels (Table 1). CD34 and CD31 are cell surface markers, and factor VIII is an essential blood clotting protein in vascular endothelial cells of normal tissues. However, these markers do not specifically identify newly formed tumor vessels, but also detect pre-existing large sized blood vessels^[17]; therefore, they are not considered suitable for the sensitive analysis of tumor angiogenesis. Inclusion of pre-existing mature vessels in MVD of various tumors may cause controversial results when determining the relationship between tumor angiogenesis, clinicopathologic features, and prognosis.

Nestin, an intermediate filament (IF) protein, has recently been recognized as a more specific marker for newly formed blood vessels and as a therapeutic target *via* inhibition of angiogenesis. In this review, we summarize and emphasize recent research evidences concerning nestin in tumor angiogenesis in GI cancers, including pancreatic cancer.

BASIC CHARACTERIZATION OF NESTIN

Nestin is a class VI IF protein originally described as a neuronal stem cell marker. After cellular differentiation, nestin expression is downregulated and replaced by neurofilament, a tissue specific IF^[18]. Nestin is also expressed in non-neuronal immature or progenitor cells in some normal tissues^[19]. It is a large protein (> 1600 amino acids) containing a short N terminus and an unusually long C terminus. The C terminus interacts with other IFs including vimentin, desmin, or internexin, subsequently forming heterodimers and mixed polymers^[19-21]. The long C-terminal portion of nestin protrudes from the filamentous body and may function as a link or cross-bridge between IFs and microtubules. Nestin is known to contribute to the disassembly of vimentin during mitosis, inactivation of cyclin-dependent kinase 5^[22], and modulation of mitosis-associated cytoplasmic reorganization during mitosis *via* phosphorylation at Thr³¹⁶ by cdc2 kinase^[23]. Neural cell-specific expression is usually regulated by the second intron, whereas nestin expres-

sion in tumor endothelium is enhanced by the first intron^[24]. Although nestin is expressed at the early stages of normal development, under pathological conditions, it is re-expressed in repair processes, various neoplasms, and proliferating vascular endothelial cells^[25].

NESTIN IN ANGIOGENESIS DURING DEVELOPMENT

Much evidence has shown that nestin expression in vascular endothelial cells is associated with proliferation and angiogenesis^[24,26-29]. Nestin expression was detected in endothelial cells of embryonic capillaries^[28], capillaries of the corpus luteum, which replenishes itself by angiogenesis^[30], and proliferating endothelial progenitor cells, but not in mature endothelial cells^[24].

At the early stage of development, high levels of nestin are identified in endothelial cells lining all blood vessels of E14-15 rat fetuses and extraembryonic (chorion, placenta, umbilical cord) and intraembryonic blood vessels. Expression of nestin by vascular endothelial cells is greatly reduced in adult tissues^[28].

These findings suggest that nestin plays important roles in vasculogenesis during development.

NESTIN IN ANGIOGENESIS IN NON-CANCEROUS DISEASES

Angiogenesis is a process of new blood vessel formation from pre-existing vessels; it is the mechanism mediating the growth and modification of a capillary network^[31]. In non-cancerous diseases, nestin plays important roles in angiogenesis of wound healing in various tissues. In an adult rat necrotizing pancreatitis model, nestin is expressed in reactive stellate cells, or submesothelial cells, and endothelial cells during active angiogenesis^[32], and nestin-positive cells may participate in tissue repair of the pancreas.

Nestin-expressing interfollicular blood vessel networks contribute to skin transplant survival and wound healing^[33]. Scar formation following an ischemic insult to the heart is referred to as reparative fibrosis and represents an essential physiological response to damaged myocardium. Several studies reported that scar formation was associated with the recruitment of neural crest-derived cardiac resident nestin-positive cells that display characteristics consistent with a neural progenitor/stem cell phenotype^[34,35]. During the reparative fibrotic response, these nestin-positive cells participate in neural remodeling and represent a novel cellular substrate of angiogenesis.

In the rat ovary, the endogenous luteinizing hormone surge induces nestin expression in capillary endothelial cells of the theca interna *via* the vascular endothelial growth factor (VEGF) signaling pathway^[36]. Angiogenic induction of chorion-derived stem cells results in higher mRNA levels of pro-angiogenic protein, fibroblast growth factor-4, and stem cell markers ABCG-2, Sox-2, FZD9, BST-1, nestin, and Oct-4^[37]. Endothelial precursors express nestin, and it participates in the formation of the cytoskeleton of newly formed endothelial cells.

Table 1 Angiogenesis markers for analysis of microvessel density

	Factor VIII	CD 31	CD 34	Nestin
Roles	Glycoprotein Blood coagulation	Glycoprotein Endothelial cell intercellular junctions ^[57] , migration	Glycoprotein Cell-cell adhesion factor, facilitate opening of vascular lumens ^[58]	Cytoskeletal protein Cell cycle ^[22,59] , migration
Character of positive vessels	Large size Mature vessels	Small to large size Immature to mature vessels	Small to large size Immature to mature vessels	Small size ^[27] Immature vessels ^[26,30] High-proliferating activity ^[26,27]
Clinical outcome				
Esophageal cancer	Poor prognosis ^[60]	Poor prognosis ^[61,62]	Poor prognosis ^[63,64]	-
Gastric cancer	Advanced stage ^[65]	No relation ^[66]	No relation ^[45]	No relation ^[44]
Colorectal cancer	No relation ^[68] Liver metastasis ^[69]	Poor prognosis ^[68]	Poor prognosis ^[67] Poor prognosis ^[68] Liver metastasis ^[69]	Poor prognosis ^[42]
Liver cancer	No relation ^[70]		Liver metastasis ^[70,72-75]	
Pancreatic cancer	Poor prognosis ^[16]	No relation ^[71] Lymph node meta ^[76]	Poor prognosis ^[77,78] No relation ^[28]	No relation ^[27]
Prostate cancer	Poor prognosis ^[79-81]	No relation ^[82]	Poor prognosis ^[83]	Poor prognosis ^[26]
Glioblastoma	Tumor grade ^[84]	No relation ^[85]	No relation ^[86]	Tumor grade ^[45]

VEGF is essential for the differentiation of the primitive embryonic vascular system and has been implicated in the vascularization of organs. VEGF is considered a modulator involved in neurogenesis as well as angiogenesis^[38,39]. Human VEGF overexpressed transgenic mice showed an increased number of nestin positive cells after temporal ischemia of the central nervous system, as compared with wild type mice^[40]. Therefore, VEGF is involved in nestin expression. Furthermore, pre-eclamptic human placenta showed higher nestin expression levels^[41], thus hypoxia may be involved in expression of nestin.

NESTIN IN TUMOR ANGIOGENESIS

Expression of nestin by newly formed blood vessels in various tumor tissues, including pancreatic cancers, has been reported^[26,27,30,42,43]. Its expression is not reported in mature vessels within cancer tissues. This suggests that nestin may be an angiogenesis-specific marker in malignant tumors^[27,44]. Strongly nestin-positive MVD colorectal carcinomas^[42] and prostate cancers^[26] show a worse prognosis. In colorectal cancer, nestin is strongly localized to vascular endothelial cells, but not to cancer cells. Nestin-expressing vessels in colorectal cancer tissues are of smaller size and show greater positivity for proliferating cell nuclear antigen than CD34-expressing cells^[42]. In glioblastomas, expression of nestin in both tumor cells and endothelial cells increased with tumor grade^[45]. Similarly, nestin expression correlates with more advanced stage in melanoma^[46]. In gastric cancers, there is no correlation between nestin-positive MVD and patient's clinical outcome, while in patients with larger carcinomas, MVD determined by nestin correlates better with longer survival than MVD determined by CD34^[44]. We previously reported that nestin-positive blood vessels in pancreatic cancer exhibit high proliferating ability, but does not correlate with prognosis^[27]. Tumor angiogenesis is essential for tumor progression, but increased angiogenesis may be influenced by variations in the existing vasculature of the host organ. Compared to MVD

studies using other endothelial markers, only a limited number of investigations have used nestin. Large scale studies of various different malignancies using nestin as a MVD marker are needed to determine the efficacy of nestin as a predictive and prognostic marker.

The origin of nestin-positive vascular endothelial cells in tumor tissue is still controversial. Migration of mesenchymal stem cells from the bone marrow has been proposed^[47]. Dong *et al.*^[48] showed that human glioma stem/progenitor cells transdifferentiate into vascular endothelial cells *in vitro* and *in vivo*. In skin angiogenesis, nestin-positive hair follicle cells form neovasculature^[49]; therefore tissue stem cells are one possible origin of nestin-positive vessels.

Recent studies showed that nestin is an effective marker for *in vivo* imaging of tumor angiogenesis^[50,51] and vascular formation in metastatic tumors^[52].

NESTIN IN PANCREATIC CANCER

Pancreatic cancer is an aggressive malignancy with an overall five-year survival rate of less than 5%^[53]. Since the majority of patients have locally advanced or metastatic disease at the time of diagnosis, there has been little progress in extending survival^[54]. For over ten years, chemotherapy with gemcitabine, whose strategy is to inhibit tumor growth, has been the standard treatment for patients with advanced pancreatic cancer, prolonging survival by only five to six months.

To improve upon this modest benefit, several investigations explored other strategies aimed at inhibiting pancreatic cancer growth, including the use of anti-angiogenic agents. Tumor angiogenesis is an important factor in the proliferation, invasion, metastasis, and drug sensitivity of pancreatic cancers^[6]. For a tumor to develop a highly malignant and deadly phenotype, it must first recruit and sustain its own blood supply. The process of angiogenesis is also sustained by secretion of specific angiogenic factors by cancer and non-cancer cells. Angiogenic factors can initiate a multi-step process that begins with vasodila-

Figure 1 Immunohistochemical images of blood vessels in pancreatic cancer. Nestin was expressed in small vessels (arrowheads) and in the surrounding cancer cells (a), but not in large vessels (arrows). CD34 and CD31 were expressed in both small and large vessels (arrowheads and arrows), whereas factor VIII was expressed only in large vessels (arrows).

tation, followed by the enhancement of vessel permeability and stroma degradation. Angiogenic factors can also promote endothelial cell migration and proliferation.

Several clinical agents have been developed to inhibit the angiogenesis process, including the VEGF pathway^[55], and a number of phase II and phase III trials combined gemcitabine with novel anti-angiogenic agents with the hope of improving patient survival. Bevacizumab, which inhibits only the VEGF receptor, and sorafenib, which inhibits raf-1 kinase and the platelet-derived growth factor receptor tyrosine kinase in addition to the VEGF receptor, were studied in combination with gemcitabine. However, this approach has largely been unsuccessful and additional novel strategies are clearly needed.

In pancreatic cancers, we found that CD34, CD31, and factor VIII, which are commonly used for MVD quantification by immunohistochemical analysis, were expressed in both newly formed small sized blood vessels and pre-existing vessels (Figure 1, arrows and arrowheads), whereas nestin was expressed only in newly formed blood vessels (arrowheads). Therefore, nestin is a potentially useful marker to distinguish newly formed blood vessels from pre-existing vessels in tumor tissues^[27].

More importantly, we observed that knockdown of nestin in vascular endothelial cells suppresses cell growth and tumor formation of pancreatic cancers *in vivo*^[27]. Therefore, nestin is a potential molecular target *via* inhibition of angiogenesis in pancreatic cancer.

Nestin is expressed in both pancreatic cancer cells and tumor vessels. Inhibition of nestin in cancer cells re-

duces tumor cell migration and metastasis^[56] and down-regulation of nestin in tumor vessels also inhibits tumor formation^[27]. These findings may indicate that nestin is a target not only for inhibition of tumor angiogenesis, but also within some cancers themselves that expression nestin, such as glioblastomas, melanomas, and pancreatic cancer (Figure 2).

CONCLUSION

More accurate estimation of MVD using nestin in GI cancers may reveal a strong relationship between tumor vessels and clinical outcome. Future studies on MVD with a large patient population are needed to clarify the efficacy of nestin as a predictive and prognostic marker. Nestin-targeting small interfering RNA (siRNA) has shown a tumor inhibitory effect *in vivo via* inhibition of tumor angiogenesis^[27], thus nestin may be a novel therapeutic target for tumor angiogenesis. Treatment resistance often occurs with the isolated use of anti-VEGF therapy. Therefore, combination therapy that also includes nestin-targeting agents is a potentially effective therapeutic strategy. Nestin is expressed in some cancer cells, thus targeted therapy may exert inhibitory effects both on cancer cells and vascular endothelium in certain tumor types, including pancreatic cancers. In addition to small molecule compounds, emerging biotechnology including RNA aptamer and siRNA targeting nestin will possibly be adopted as new therapeutics for GI cancers, especially for pancreatic cancers.

Figure 2 In pancreatic cancer, nestin is highly expressed in some pancreatic cancer cells and small proliferating tumor vessels.

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