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REVIEW ARTICLE

Current insights into the association of Nestin with tumor angiogenesis

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Summary

Tumor angiogenesis is regarded as a hallmark of cancer and provides an important target for therapy. Nestin is an intermediate filament protein (IF) originally recognized as a neural stem cell marker. Development and progression of cancer requires sustained angiogenesis, dependent on the proliferation and migration of endothelial cells which seem to be better portrayed by nestin expression in various malignancies such as central nervous system, gastro-intestinal cancers, malignant melanoma, lung, prostate or breast cancer.

The purpose of the present review was to emphasize the insights into nestin expression in relation to tumor angiogenesis in different types of cancer. Current evidence suggests that nestin positivity in tumor cells reflects stem-like properties of those cells. Whether or not expressed in both tumor and endothelial cells, nestin overexpression might reflect the extent of angiogenesis and function as a molecular anti-angiogenic target for cancer.

Key words: *cancer stem cells, microvascular density, nestin, tumor angiogenesis*

Introduction

Tumor neovascularization relies on two non-mutually exclusive events: angiogenesis and vasculogenesis. Whatever the case, complex interactions between tumor-secreted factors and various cell types nourish an angiogenic cascade within the tumor microenvironment, upholding vessel formation [1,2].

Regarded as a hallmark of cancer, the degree of angiogenesis in various cancer sites has been extensively assessed through microvascular density (MVD) by means of endothelial markers such as CD34, CD31 and factor VIII [3,4]. Although the prognostic significance of MVD has been certified in several studies, controversies over this issue are still under debate [5,6]. One major drawback might be that these commonly used vascular markers detect not only newly formed microvasculature but also preexisting, mature tumor vessels [5,7].

Nestin is an IF formerly used to depict neuroepithelial stem cells. Arguments that nestin might be actively involved in the modulation of the cytoskeleton [8], strengthen the idea that nestin is not just a structural protein, but an active participant to cellular processes, by coordinating cell dynamics, adhesion and migration [8,9]. It is hypothesized that nestin mirrors the properties of cancer stem cells (CSCs) in different tumors, correlating with poor prognosis [9-11]. Hence, nestin is recognized as a marker of immature, undifferentiated cell populations that specifically exhibit capacities like regeneration, proliferation and mi-

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gration [5,12].

Several studies proposed nestin as a reliable marker for proliferative endothelial cells in tissues undergoing neovascularization [12-14]. Thus, endothelial expression patterns of nestin have been observed in various pathologic conditions such as brain injury, ischemia, inflammation and cancer [5,14]. The first intron of nestin gene seems to be responsible for nestin endothelial-specific expression [15].

In order to provide an up-to-date review on nestin expression in relation to tumor angiogenesis in different cancer sites, we comprehensively searched PubMed and Web of Science electronic databases for relevant articles published between 2002 and 2014. Search terms as nestin, tumor angiogenesis, microvascular density, cancer stem cells, revealed 139 articles. With appropriate selection, 57 studies were considered.

The aim of this review was to discuss current evidence that reveals the role of nestin as a potential marker for tumor neoangiogenesis (Table 1). In addition, the potential therapeutic significance of targeting nestin is envisaged.

Central nervous system (CNS) cancer

Being a marker of neural stem cells, nestin has been reported to be relevant for the identification of CSCs [16] and a reliable endothelium marker for CNS tumors [17-19]. According to Calabrese et al, the proximity of nestin-positive cells to tumor microvasculature in medulloblastoma, ependymoma, oligodendroglioma, glioblastoma, advocates the existence of a vascular niche to assure the essential microenvironment for CSCs survival, proliferation and tumor growth [20,21].

Above all, nestin overexpression has been particularly detected in high grade gliomas, correlating with vascular endothelial growth factor (VEGF) expression and endothelial cell proliferation into a glomeruloid vascular pattern, reflecting higher angiogenic activity, tumor invasiveness and poorer survival [18,19,22]. Among high grade gliomas, nestin has been constantly overexpressed in glioblastomas (GBMs) [18], one of the most lethal forms of cancer due to its aggressive behavior [21].

As tumor progression seems to be dependent on the degree of neoangiogenesis in both tumor and surrounding tissues [21,23], Sica et al. (2011) analyzed the vascularization in peritumoral areas of 40 GBM tissue samples through immunohistochemical (IHC) examination of MVD determined by nestin and CD105 (endoglin). Irrespective of the presence of malignant cells, similar nestin-positive microvascular patterns have been detected in the tissues adjacent to tumor margins (<1cm) as well as in more distant peritumoral areas (1-3.5 cm), compared to CD105 expression that progressively decreased with distance from the tumor margins. It is suggested that nestin overex-

Table 1. The association of nestin with tumor angiogenesis in different cancer types

Type of cancer	*First author (year)*	*Ref.*	*No. of cases*	*Nestin expression*
Glioblastoma	Mangiola et al. (2007) Sica et al. (2011) He et al. (2012)	[24] [23] [27]	20 40 70	angiogenesis at the tumor invasion front differentiation of GSCs –newly formed vessels
Astrocytoma	Hlobilkova et al. (2009)	[30]	66	angiogenesis activation in high grade tumors
Ependymoma	Nambirajan et al. (2014)	[28]	126	endothelial proliferation, higher microvessel density
Melanoma	Brychtova et al. (2007) Piras et al. (2010)	[31] [33]	139 152	endothelial expression – prognostic value
Gastric cancer	Kim et al. (2002)	[6]	61	higher microvessel density
Colorectal cancer	Teranishi et al. (2007)	[36]	101	small size, proliferating vessels
Pancreatic cancer	Yamahatsu et al. (2012)	[37]	45	
HCC	Yang et al. (2010)	[47]	67, 73 314	angiogenesis-related biomarker
NSCLC	Chen et al. (2010) Ahmed et al. (2014)	[40] [41]	52 27	increased lymphangiogenesis proangiogenic capacities
Prostate cancer	Gravdal et al. (2009)	[42]	104	immature, proliferating microvessels
Breast cancer	Krüger et al. (2013)	[44]	178	
Ovarian cancer	Qin et al. (2012)	[48]	123	putative relation to tumor angiogenesis

HCC: hepatocellular carcinoma, NSCLC: non-small cell lung cancer, GSCs: glioblastoma stem cells

pression in these peritumoral areas activates endothelium, which afterwards acquires malignant properties reflected by CD105-MVD [23]. Similarly, Mangiola et al. reported that activation of the endothelium in the tumor invasion front revealed by activated Jun NH2-terminal kinases (pJNK)/Nestin expression, can significantly influence GBM prognosis and survival [24]. Hence, quantifying neovascularization in peritumoral areas might add prognostic relevance to intratumoral angiogenesis.

The close interactions between glioblastoma stem-like cells (GSCs) and the so-called vascular niche, supports the idea that these cells can also behave like endothelial progenitors and are able to transdifferentiate into endothelial cells. By generating new vasculature, GSCs contribute directly to vasculogenesis mechanisms. What's more, GSCs release angiogenic factors leading to increased angiogenesis [25,26].

In accordance with these hypotheses, He et al. (2012) analyzed 70 glioblastoma samples by IHC and double immunofluorescence staining and showed a perivascular distribution of GSCs, as both the CD133-positive cells and nestin-positive cells have been concentrated around the CD31-positive blood vessels. More than that, nestin positivity in the endothelial cells of tumor vasculature has been strongly associated with nestin positivity in glioblastoma cells [27]. These facts sustain that bidirectional interactions might exist between these two compartments and might lead not only to increased vascularity of the tumor, but also to a putative substrate for therapy resistance and new targeted therapies [25].

When focusing on less frequent glial tumors like ependymomas, Nambirajan et al. (2014) have correlated nestin overexpression with endothelial proliferation, higher CD34-determined MVD, as well as elevated VEGF expression, altogether certifying the augmented neoangiogenesis process [28]. What's more, nestin alongside with VEGF might help distinguish between site-specific ependymomas, being undoubtedly more expressed in supratentorial ones, which have the most aggressive phenotype, are frequently poorer differentiated, and have shorter progression free survival (PFS), when compared to the infratentorial and spinal ependymomas [28,29]. However, endothelial expression of nestin needed corroboration with nestin expression in tumor cells in order to achieve a strong association with VEGF [28], which apparently supports the putative interaction between CSCs and the vascular niche, the latter being essential for CSCs survival, self-renewal and tumor growth [20]. Similar interplay between nestin expression in tumor and endothelial cells and VEGF has been suggested in astrocytomas, especially in high-grade ones, contributing to activation of angiogenesis and migration and influencing survival [30]. Targeting the vascular niche through VEGF inhibitors might disturb the above mentioned mechanism and result in treatment strategies optimization [20].

Malignant melanoma

Brychtova et al. (2007) immunohistochemically analyzed 139 melanocytic tumor samples and found that nestin was significantly overexpressed in the capillary endothelium adjacent to advanced malignant melanoma tumors, with more than 1 mm in thickness, compared to early-stage tumors ($p<0.01$)[31]. The depth of dermal invasion is a well-known prognostic factor [32] and association with nestin overexpression in less differentiated, immature endothelial cells might certify a more dynamic angiogenic stimulation within tumor microenvironment [31].

Further evaluation of a series of 152 melanomas identified nestin positivity in endothelial cells of microvessels in almost 48% of primary tumors and 50% of nodal metastases [33]. Piras et al. (2010) reported that nestin positivity in both endothelial cells and tumor cells seems to amplify its predictive value for both early and advanced stages of melanoma, reflected in significantly reduced overall survival (OS). Likewise, Brychtova et al. have observed increased nestin-positive tumor cells and microvessels in the peripheral areas of primary tumors [33], where the invasive and proliferation capacities of less differentiated malignant cells seem to be more prominent. Moreover, in this area neovasculature might be facilitated by endothelial precursor cells expressing nestin, forming the cytoskeleton of new endothelial cells and consecutive immature, leaky vessels that foster tumor growth and metastasis [13].

A recent study has strengthened the key role of nestin as a marker for melanocyte stem cells (using CD133 as control) as well as a marker of endothelial proliferation in malignant melanoma. The latter has been confirmed by the strong correlation between the extent of angiogenesis (reflected through CD34-determined MVD) and nestin expression in the endothelium of microvessels [34].

Gastrointestinal cancers

Tumor angiogenesis represents a remarkable pathway involved in tumor progression and metastasis [35]. Although MVD has been extensively used for neoangiogenesis assessment in gastrointestinal cancers [5], better angiogenesis markers are needed for less contrasting results regarding prognostic significance.

Following this hypothesis, nestin staining has been detected in the cytoplasm of most vascular endothelial cells, next to cancer cells in gastric and colorectal adenocarcinomas, with increased immunoreactivity at the tumor invasion front into the surrounding tissues [6,36]. Nestin-determined MVD was significantly higher and strongly associated with CD34-determined MVD in gastric adenocarcinoma ($p<0.001$) [6]. In colorectal cancer tissues, nestin has been identified in endothelial cells of small blood vessels (6.30 μm median diameter), whereas CD34 also immunostained larger, lumen-formed vessels (8.82 μm median diameter). Moreover, the significant correlation of nestin expression with endothelial cell proliferation ($p=0.002$), appreciated by means of a proliferating cell nuclear antigen (PCNA) expression, emphasizes the ability of nestin to detect newly-formed, proliferating microvessels in colorectal and pancreatic adenocarcinomas. *In situ* hybridization of nestin mRNA came as a validation of the above-mentioned, strengthening the putative implication of nestin in colorectal cancer development through angiogenesis [36,37].

Even though nestin showed superiority in the evaluation of neovascularization in these gastrointestinal cancers, no associations with clinicopathological factors have been achieved. For gastric adenocarcinomas larger than 5cm, a significant survival difference has been observed ($p=0.032$) [6]; for colorectal cancer, more modest results in terms of PFS have been observed in patients with higher rates of MVD determined by nestin than by CD34, implying that besides being a marker for neovascularization, nestin might become a valuable prognostic factor [36].

Although nestin expression in pancreatic cancer vessels hasn't proved a significant impact on clinical outcome, the specific role of nestin has been further deciphered in pancreatic cancer cell lines. A gene-silencing strategy based on small interfering RNA (siRNA) targeting nestin, has been used to downregulate nestin and a significant inhibition of vascular endothelial cells growth and tumor formation *in vivo* has been achieved [37,38]. Altogether, besides the role of nestin as a novel angiogenesis biomarker, considering it a potential target could represent a promising antiangiogenic approach in pancreatic adenocarcinoma.

Non small cell lung cancer (NSCLC)

In NSCLC nestin positivity has been confirmed in tumor cells and vascularized tumor areas of both primary tumor, lymph node and brain metastasis, with higher density of nestin-positive microvessels in advanced and metastatic stages [39-41].

In this context, Chen et al. (2010) reported that cells overexpressing nestin may be responsible for enhanced lymphangiogenesis in NSCLC tumor samples and subsequent shorter survival time ($p=0.005$). Nestin positivity has been associated with poor differentiation, adenocarcinoma histologic subtype, N2 lymph node metastasis, higher lymphatic vessel density (LVD) and higher MVD determined by CD34 and VEGFR-3, albeit the mismatch between VEGF/VEGF-C levels and nestin expression has been unexpected [40].

Following these hypotheses, Ahmed et al. (2014) used RT-PCR in 27 lung adenocarcinoma biopsies and significantly related nestin expression with high histologic grade, advanced stage and serum VEGF, assuming its putative role in tumorigenesis and neoangiogenesis, through immature cells proliferation and proangiogenic capacities [41].

Other cancers

Nestin predilection for the endothelial cells of small, immature tumor vessels has been also noticed by IHC analysis of prostate cancer specimens, in both primary tumors and bone metastases [42,43]. Gravdal et al. (2009) have proposed a novel, more trustworthy angiogenesis dual-marker, defined by co-expression of nestin and ki67, aiming to reflect the proliferation capacities of the nestin-positive microvessels. This combination, a potential hallmark of the actively expanding vasculature, has been strongly associated with VEGF-A expression, showing significant predictive value for disease progression, biochemical failure, locoregional recurrence and bone metastases in localized carcinomas. Furthermore, increased nestin/ki67 immunoreactivity has been detected in castration-resistant prostate cancer and metastatic lesions, with diminished survival rates in these patients [42]. Eventually, these observations might help in predicting resistance to treatment and identifying a subgroup with poor

prognosis specifically due to vascular proliferation, followed by amplified migration and invasion processes.

A similar judgment, based on nestin and ki67 expression, has been used to better quantify tumor angiogenesis in breast carcinomas, reflecting the proliferating, immature microvessels. Microvessel proliferation, expressed as a vascular proliferation index (VPI), has been established as an independent prognostic factor significantly associated with survival and some unfavorable tumor characteristics (negative ER and PR status, higher proliferation rate in tumor cells, and p53 positivity) [44]. Furthermore, activated angiogenesis appreciated through nestin and ki67 overexpression, has been more pronounced in basal-like, triple-negative breast cancer compared to the other phenotypes [44], emphasizing the few treatment options available for this subtype and the opportunity for new therapeutic strategies targeting angiogenesis.

As already mentioned, nestin has been advocated in several studies as being member of a panel of biomarkers expressed on a subpopulation of cells with remarkable "stemness" properties [1,45]. Besides their putative role in tumorigenesis and therapeutic resistance, cancer stem cells seem to contribute to tumor angiogenesis by induction of various proangiogenic factors, transdifferentiation into endothelial cells and vascular smooth muscle-like cells, forming the nonendothelium-lining vascular mimicry [45,46].

In order to highlight the links between tumor angiogenesis and CSCs expression profile Yang et al. (2010) analyzed hepatocellular carcinoma (HCC) samples using qRT-PCR and IHC, and found that this subpopulation of cells, revealed by a panel of putative biomarkers, including nestin, has been significantly related to higher CD34-determined MVD and VEGF levels, as well as lower PFS and OS. These arguments might confirm CSCs implications in HCC angiogenesis process through proangiogenic factors release. Predictive models based on the degree of neovascularization and a set of several CSCs biomarkers, like nestin, CD133 and CD44, could be useful for a superior prediction of the clinical outcome [47].

This theory seems to be also suitable for advanced serous ovarian carcinoma. The significant association of nestin expression with tumor angiogenesis (reflected by VEGF levels and CD34-determined MVD) sustains the potential implication of nestin-positive cells in ovarian cancer progression through the angiogenesis process [48]. Moreover, nestin seems to have a predictive value for chemotherapy response. Altogether, these arguments strengthen the idea that CSCs reflected by nestin expression might be responsible for more aggressive behavior and reduced sensitivity to chemotherapy, thus selecting those patients who should benefit of more complex therapeutic strategies [48,49].

Angiogenesis imaging and therapeutic perspectives

Accumulating evidence indicates that tumor angiogenesis can be imaged with fluorescent proteins in experimental mouse tumor models. Nascent blood vessels have been quantified by means of nestin positivity in proliferating endothelial cells [50,51], highlighting neoangiogenesis for human lung cancer, pancreatic cancer, colon cancer, human glioma, murine melanoma and breast cancer cell lines, bone and soft tissue sarcoma [51-53]. Besides these primary tumors, similar models of vascular formation have been also observed in liver and lung metastatic tumors of melanoma, as well as in liver metastasis of human pancreatic cancer [54,55]. In addition, downregulation of nestin expression has been achieved using conventional [53,56] or targeted agents [20,57], as well as siRNA targeting nestin [38], with significant inhibition of angiogenesis and tumor growth. Hence, novel angiogenic inhibitors could be screened and evaluated through similar nestin-expressing models.

Although an association between nestin and VEGF expression during angiogenesis has been established, the regulatory mechanism behind this relationship needs clarification. Liang et al. (2014) reported that nestin-mediated cytoskeleton remodeling promotes endothelial cells migration via VEGF induction and that VEGF-induced upregulation of nestin in endothelial cells seems to be mediated by ERK signaling pathway. Inhibition of nestin expression has significantly reduced the events associated with VEGF-induced angiogenesis, especially the migration of endothelial cells [14]. These facts support its role in angiogenesis and suggest a potential target.

Effective therapeutic strategies in order to overcome resistance to therapies would be one more imperative step forward. In this respect, *in vitro* treatment attempts such as arsenic trioxide in combination with conventional chemoradiotherapy for glioblastoma multiforme, might defeat the tumorigenic properties of glioblastoma stem-like cells (recognized through cellular markers like

nestin, CD133, CD105, Nanog, Oct3/4, CXCR4) and enhance chemo- and radiosensitivity, leading to potential outcome benefits [58,59].

Conclusion

Although originally acknowledged as a neural stem cell marker, extensive research showed that nestin is also expressed in endothelial cells of tumor vessels as well as in rapidly proliferative cells of various malignant tissues, reflecting stem-like properties.

In different cancer sites, nestin-determined MVD seems to better reveal the early phases of neovascularization being a more sensitive marker for activated, undifferentiated endothelium of newly-formed vessels. Furthermore, knowledge of the proliferating status of tumor microvessels refines the prognostic value of nestin-MVD. However, only a limited number of investigators have used nestin for tumor angiogenesis estimation, with inconsistent results with regard to its prognostic significance. Larger studies of various malignancies are needed for further validation.

Given its association with VEGF-induced angiogenesis, nestin might represent a promising therapeutic target as well as a useful predictive biomarker of efficacy and response to current antiangiogenic therapies that focus on VEGF or its receptors [14]. Since tight interplay between CSCs and tumor angiogenesis is presumed, treatment approaches targeting CSCs with simultaneous inhibition of tumor angiogenesis might open new perspectives for more appropriate personalized strategies.

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