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Angiogenin-mediated ribosomal RNA transcription as a molecular target for treatment of head and neck squamous cell carcinoma

Lili Chen^{a,*} and Guo-fu Hu^{b,*}

^aDepartment of Stomatology, Wuhan Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan, 430022, China

^bDepartment of Pathology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA

SUMMARY

Squamous cell carcinoma of the head and neck (HNSCC) is the eighth most common disease, affecting approximately 640,000 patients worldwide each year. Despite recent advances in surgery, radiotherapy, and chemotherapy, the overall cure for patients with HNSCC has remained at less than 50 percent for many decades. Patients with recurrent and metastatic disease have a median survival of only 6–10 months. Systemic chemotherapy is the only treatment option for those patients. New treatment options are thus desperately needed to supplement, complement, or replace currently available therapies. New agents that target molecular and cellular pathways of the disease pathogenesis of HNSCC are promising candidates. One class of these new agents is angiogenesis inhibitors that have been proven effective in the treatment of advanced colorectal, breast, and non-small cell lung cancers. Similar to other solid tumors, angiogenesis plays an important role in the pathogenesis of HNSCC. A number of angiogenic factors including vascular endothelial growth factor (VEGF) and angiogenin (ANG) have been shown to be significantly upregulated in HNSCC. Among them, ANG is unique in which it is a ribonuclease that regulates ribosomal RNA (rRNA) transcription. ANG-stimulated rRNA transcription has been shown to be a general requirement for angiogenesis induced by other angiogenic factors. ANG inhibitors have been demonstrated to inhibit angiogenesis and tumor growth induced not only by ANG but also by other angiogenic factors. As the role of ANG in HNSCC is being unveiled, the therapeutic potential of ANG inhibitors in HNSCC is expected.

Keywords

Angiogenin; angiogenesis; HNSCC; rRNA transcription

Head and neck cancers

Head and neck cancers are the malignancies that arise from the mucosal epithelia of the oral cavity, nasal cavity, pharynx, and larynx.¹ It is thus a heterogeneous disease with various

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*Corresponding authors. Tel: +1 617-432-6582; fax: +1 617-432-6580. lily-c1030@163.com (L. Chen), ghu@hms.harvard.edu (G. Hu).

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histological presentations and differentiation patterns. The most common form is squamous cell carcinoma (SCC), which accounts for more than 90% of all the head and neck cancer cases. The risk factors of HNSCC are well understood. At least 75% of HNSCC can be attributed to a combination of cigarettes smoking and alcohol drinking.² High risk types of human papillomavirus (HPV), in particularly HPV-16, also contributes to a subgroup of HNSCC.³ Like other types of cancers, HNSCC is also believed to arise via a multistep process involving the activation of oncogenes as well as the inactivation of tumor suppressor genes. Mutations of the tumor suppressor *P53*, one of the most frequently altered gene in human cancers, have also been shown to be associated with HNSCC.⁴ *P53* mutations are not only an underlying mechanism of cancer initiation and development, but also often result in gain-of-function effects causing resistance to radiotherapy and chemotherapy.⁵ Inactivation of cell cycle inhibitor p16, caused by homozygous deletion, point mutations, or promoter hypermethylation, have been documented in HNSCC.^{6, 7} In contrast, cell cycle protein cyclin D1 has been shown to be overexpressed.^{8, 9} Moreover, multiple genetic aberrations including DNA copy number variations and loss of heterozygosity have also been shown to have an impact on HNSCC.¹⁰ Regions in the chromosome where oncogenes are located are in general amplified.² Besides genetic aberrations that predispose to HNSCC initiation, upregulation of angiogenic factors such VEGF and ANG have also been shown to significantly contribute to the development of HNSCC.^{11, 12}

Current therapy of HNSCC

Treatment decisions in HNSCC are often complicated by the anatomical location and desire to keep organ preservation thus maintaining certain level of quality of life. Early stage HNSCC patients are usually treated with surgery, radiotherapy, chemotherapy or the combination of these modalities.^{13, 14} However, approximately half of the patients will develop local, regional or distant relapses, which usually occur within the first 2–5 years of treatment.² Multiple reasons contribute to the high recurrence rate of HNSCC. First of all, the location of the HNSCC prevents the surgeon from gaining complete locoregional control of the primary lesion. Second, HNSCC very often occur in multiple primary lesions, which significantly complicate surgical resection of primary tumors. Moreover, HNSCC has a propensity of regional metastasis to the cervical lymph nodes, thereby facilitating systemic metastasis. Prognosis of these recurrent patients is very poor with a median survival of only 6–10 months. The only treatment option for recurrent HNSCC is systemic chemotherapy that has a particularly intolerable toxicity to HNSCC patients who usually have problematic lifestyles and various morbidity problems.¹⁵ Additional treatment options with improved efficacy and lower toxicity are thus urgently needed for HNSCC. Unfortunately, few adjunct therapies have yet offered significant survival benefit for HNSCC patients, which has remained unchanged for many decades.

Angiogenesis as a molecular target for HNSCC drug development

As the mechanism of HNSCC initiation, progression, invasion, spread, and distant metastasis are becoming unveiled, new opportunities arise for targeted intervention. Agents that specifically target these cellular and molecular pathways associated with HNSCC are promising candidates as they are already successfully used in other neoplasia such as colorectal cancer, lung cancer, breast cancer, and hematological malignancies.¹⁶ The metastatic process of HNSCC appears to be similar to that of other solid tumors, which are characterized with a sequential process of local invasion, intravasation, circulating, extravasation, and recolonization and growth in distant organs. HNSCC lesions are generally very vascular, and have enhanced lymphatic vasculature to facilitate drainage from these area.¹⁷ Therefore, one of the effective pathways to target for HNSCC therapy will be tumor angiogenesis.

Angiogenesis

Angiogenesis is a process by which endothelial cells migrate, proliferate, and organize to form new blood vessels.¹⁸ It is essential for various physiological processes, including reproduction, development and wound repair. It also features in many pathological conditions such as tumor growth and metastasis, arthritis and diabetic retinopathy.¹⁹ Angiogenesis is a multistep process controlled by the net balance between stimulators and inhibitors.²⁰ For example, tumor angiogenesis has been shown to include: (i) sprouting, (ii) intussusception, (iii) formation of extended 'mother vessels', (iv) 'splitting' of mother vessels and formation of 'daughter vessels', (v) vascular fusion, (vi) recruitment of circulating endothelial progenitor cells, (vii) cooption and modification of pre-existing blood vessels, and (viii) inclusion of tumor cells into the walls of vascular channels.^{21, 22} It is now well understood that normally quiescent endothelial cells become invasive and protrude into the perivascular tissues in response to angiogenic stimuli.²³ As the endothelial cells sprout, proteases are activated causing the surrounding basement membrane to lyse, allowing the cells to migrate.²³ The adjacent cells divide to occupy the space created by the migrating cells. By a continuous process of penetration, migration, proliferation and differentiation, the endothelial cells eventually form a new capillary network.²⁴ Smooth muscle cells are subsequently recruited to migrate along the newly formed endothelium. They proliferate and deposit extracellular matrix components for the formation of vessel walls²⁵ and interact with endothelium to make a complete lining²⁶. There are therefore many molecular players in the process of angiogenesis. A concept of "angiogenic switch" referring to the onset of tumor angiogenesis, which is triggered by a surplus of endogenous angiogenic stimulators over inhibitors, has been proposed to describe a seemingly distinct event in tumor progression²⁷.

Angiogenic factors

It is generally believed that the 'angiogenic switch' in cancer is the result of a change in balance between angiogenic stimulators and inhibitors present at the site of tumor growth.²⁸ Numerous angiogenic stimulators have been identified and their expression and distribution have been associated with angiogenesis-based diseases (table 1).

Although these angiogenic factors have very diverse biological and biochemical properties, they share some common properties such as inducing proliferation and migration of blood vessel cells (endothelial cells and smooth muscle cells). The signal transduction pathways of these angiogenic factors are more or less understood now. No matter how diverse the signaling pathways might be for these various angiogenic factors, their actions all resulted in sustained cell growth and proliferation. They therefore all require the production of ribosomes, which are the factories for protein translation. Ribosomal biogenesis is a process involving rRNA transcription, processing of the pre-rRNA precursor and assembly of the mature rRNA with ribosomal proteins⁴⁸. It has been known that the production of ribosomal proteins is mediated by the mTOR-S6K pathway that can be activated by upstream kinases including AKT and Erk. Many of the angiogenic proteins listed in Table 1 are known to activate mTOR and its downstream target S6K. S6 phosphorylation has been associated with translation of a specific class of mRNA termed TOP (a terminal oligopyrimidine track in the 5' untranslated region) mRNA. This class of mRNAs includes ribosomal proteins, elongation factors 1A1 and 1A2, and several other proteins involved in ribosome biogenesis or in translation control. Therefore, it is conceivable that these angiogenic proteins will stimulate the synthesis of ribosomal proteins. However, it had remained unclear how transcription of rRNA is proportionally enhanced. Recently advancement has pointed out that rRNA transcription in endothelial cells upon stimulation of various angiogenic factors is mediated by ANG.^{49–52} ANG-mediated rRNA transcription has been shown to be a general requirement for angiogenesis, which is a crossroad in the process of angiogenesis for a variety of angiogenic factors.⁵⁰ ANG inhibitors

have recently in the spotlight for anti-angiogenesis research as they inhibit angiogenesis regardless of the nature of stimuli.

ANG

ANG was isolated in 1985 from the conditioned medium of HT-29 human colon adenocarcinoma cells based on its angiogenic activity.⁵³ Structure/function studies have shown that the 123-residue protein contains a ribonucleolytic active site, a cell binding site and a nuclear localization sequence (NLS). Thus, ANG is a ribonuclease whose weak but characteristic ribonucleolytic activity⁵⁴ is essential for angiogenesis.⁵⁵ It is pleiotropic toward endothelial cells: it binds to the cell surface⁵⁶, interacts with a 170 kDa receptor⁵⁷ or a 42 kDa binding protein⁵⁸ on the cell surface, induces cell proliferation,⁵⁷ activates cell-associated proteases⁵⁹ and stimulates cell migration and invasion.⁶⁰ It also mediates cell adhesion⁶¹ and promotes tube formation of cultured endothelial cells.⁶² All of these individual cellular events are considered necessary components of the process of angiogenesis. It is also known that ANG undergoes nuclear translocation⁶³ by a process that is independent of lysosomes and microtubules.⁶⁴ Nuclear accumulation of ANG is essential for its biologic activity. When nuclear translocation is inhibited, its angiogenic activity is abolished.⁶⁵

Recently, ANG has been shown to bind to the promoter region of ribosomal DNA (rDNA) and stimulate rRNA transcription.⁶⁶ An ANG binding DNA sequence has been identified and has been shown to have ANG-dependent promoter activity in a luciferase reporter system.⁶⁷ Thus, unlike other angiogenic factor, ANG stimulates rRNA transcription directly. More importantly, ANG-mediated rRNA transcription in endothelial cells is necessary for angiogenesis induced by other angiogenic molecules.⁵⁰ In other word, ANG is a permissive factor for other angiogenic factors to induce angiogenesis due to its unique role in mediating rRNA transcription that is essential for cell growth and proliferation.

rRNA synthesis and cell growth

Regulation of protein synthesis is an important aspect of growth control. When cells are quiescent, the overall rate of protein accumulation is reduced. On mitogenic stimulation the synthesis of rRNA, ribosomal proteins and translation factors is accelerated and protein production increases before cells reach S phase.⁶⁸ The rate of growth is directly proportional to the rate of protein accumulation and this is related to ribosome content.⁶⁹ As ribosome biogenesis is a limiting factor for cell duplication, the rate of cell proliferation could be controlled by modulating the expression of nucleolar proteins involved in rRNA transcription, processing, and transport to the cytoplasm. rRNA transcription can also be regulated at the level of nuclear localization of those proteins that are synthesized in the cytoplasm or by nuclear translocation of exogenous proteins that are somehow involved in rRNA transcription. Recently reports have demonstrated that ANG is one of these proteins.^{49–51, 63, 64, 66, 67, 70–72} As the rate-limiting step in ribosome biogenesis is the synthesis of rRNA, inhibition of rRNA synthesis would then be an effective means to control cell growth, irrespective of growth stimuli.^{49, 70, 72}

ANG in HNSCC

It has been demonstrated that serum ANG concentrations are elevated in patients with various types of cancers including astrocytoma,⁷³ breast carcinoma,⁷⁴ cervical cancer,⁷⁵ colonic adenocarcinoma,⁷⁶ colorectal cancer,⁷⁷ endometrial cancer,⁷⁸ gastric adenocarcinoma,⁷⁹ gynecological cancer,⁸⁰ head and neck squamous cell carcinoma,^{81, 82} leiomyosarcoma,⁸³ lymphangioma,⁸⁴ myeloma,⁸⁵ hepatocellular carcinoma,⁷⁶ leukemia (AML, MDS),⁸⁶ lymphangioma,⁸⁷ lymphoma (non-Hodgkin's),⁸⁸ melanoma,⁸⁹ osteosarcoma,⁹⁰ ovarian cancer,⁹¹ pancreatic cancer,⁹² prostate cancer,⁹³ renal cell carcinoma,⁹⁴ urothelial carcinoma,

⁹⁵ and Wilms tumor.⁹⁶ The implication of an elevated ANG level is that tumors need rampant angiogenesis. Several animal models have been established to examine the anti-angiogenesis and subsequent anticancer activity of ANG antagonists. Most of the previous efforts have been focused on prostate cancer, breast cancer, and colorectal cancer.^{49, 51, 52, 70, 72, 97–100} The role of ANG in HNSCC is a less explored area. However, several compelling reasons suggest that ANG plays an important role in HNSCC and that ANG inhibitors are plausible candidates as novel therapeutic agents for the treatment of HNSCC. First, ANG expression is significantly elevated in HNSCC.^{81, 82} Second, there is profuse tumor angiogenesis in HNSCC tissues¹⁷ and VEGF, another prominent angiogenic factor, is also highly upregulated in HNSCC.^{101–106} Third, ANG is a permissive factor for other angiogenic factors to induce angiogenesis.⁵⁰ Thus, ANG inhibitors will also inhibit VEGF-induced angiogenesis.

ANG as a molecular target for cancer drug development

The essential role of ANG in mediating rRNA transcription in endothelial cells suggests that ANG is a molecular target for drug development. Both ANG and its receptor can be targeted for this purpose. Proof of concept has been established for targeting ANG itself as ANG-specific siRNA and antisense that inhibit ANG synthesis, and monoclonal antibody (mAb) and binding proteins that neutralize secreted ANG proteins have all been shown to inhibit xenograft growth of human cancer cells in athymic mice.^{52, 98, 99} One caveat of this strategy is the relatively high circulating ANG protein (~250–350 ng/ml) in plasma.^{92, 95} The majority of the circulating ANG is produced by the liver.¹⁰⁷ Moreover, with a seemingly fast turnover rate and a half-life of 2 h,¹⁰⁸ a large quantity of ANG inhibitors would be needed to neutralize the circulating ANG.

The cell surface receptor of ANG has not yet been identified. Therefore, targeting ANG receptor and its signaling pathway is currently not feasible. However, blockage of nuclear translocation of ANG seems to be a promising approach to inhibit the function of ANG. The biological function of ANG is related to rRNA transcription,⁶⁶ which requires ANG to be in the nucleus physically.⁶⁷ Nuclear translocation of ANG is essential for its biological function.⁶³ Targeting nuclear translocation of ANG would avoid potential problems caused by its high plasma concentration. Another distinct advantage of targeting nuclear translocation of ANG would be that it might not have serious side effects since nuclear translocation of ANG occurs only in proliferating endothelial and cancer cells.^{50–52}

Inhibitors of nuclear translocation of ANG

In efforts to understand the mechanism by which ANG is translocated to the nucleus of endothelial cells, neomycin, an aminoglycoside antibiotic, was discovered to block nuclear translocation of ANG and to inhibit ANG-induced cell proliferation and angiogenesis⁶⁵. Moreover, neomycin has been shown to inhibit xenograft growth of human cancer cells in athymic mice⁵² as well as AKT-induced prostate intraepithelial neoplasia (PIN) in AKT transgenic mice.⁴⁹ Neomycin is an FDA-approved antibiotic originally isolated from *Streptomyces fradiae*.¹⁰⁹ Similar to other aminoglycosides, neomycin has high activity against Gram-negative bacteria, and has partial activity against Gram-positive bacteria. However, neomycin is nephro- and oto-toxic to humans and its clinical use has been restricted to topical preparation and oral administration as a preventive measure for hepatic encephalopathy and hypercholesterolemia by killing bacteria in the small intestinal tract and keeping ammonia levels low.¹¹⁰ The nephro-toxicity of neomycin is associated with selective accumulation in the kidney where the cortical levels may reach as high as 20 times those of circulating levels in serum. The mechanism underlying selective renal accumulation has been shown to be tubular re-absorption, extraction from the circulation at the basolateral surface, as well as brush border uptake.¹¹¹ The antibiotic activity and the renal toxicity of neomycin seem to be separable from

its capacity to inhibit nuclear translocation of ANG. This has led a search for less toxic derivatives and analogues of neomycin and led to the finding that neamine,¹¹² a virtually nontoxic derivative of neomycin, has comparable activity in blocking nuclear translocation of ANG.⁷⁰ Neamine is equally effective in inhibiting angiogenesis and tumor growth induced by ANG as well as by other angiogenic factors.^{70, 72} Other aminoglycoside antibiotics including streptomycin, gentamicin, kanamycin, amikacin, and paromomycin do not block nuclear translocation of ANG and are not anti-angiogenic.⁶⁵

Neamine is a degradation product of neomycin although there is some evidence that it is also produced in small amounts by *Streptomyces fradiae*.¹¹² Cell and organ culture experiments have shown that the nephro- and oto-toxicity of neamine is ~5 and 6%, respectively, of that of neomycin.^{111, 113} Thus, the toxicity of neamine is similar to that of streptomycin, an antibiotic that is currently in clinical use. Neamine is also less neuromuscularly toxic than neomycin. The acute LD₅₀ (subcutaneous) in mice for neamine, neomycin, and streptomycin is 1,250, 220, and 600 mg/kg, respectively.¹¹⁰ Neamine appears to be less toxic than streptomycin.¹¹⁴

Perspective

When Avastin (bevacizumab), an anti VEGF monoclonal antibody, was approved by FDA in 2004 for the treatment of advanced colorectal cancer, angiogenesis inhibitors were declared as the fourth modality for cancer treatment. Avastin has also been approved in 2008 by FDA for the treatment of recurrent and metastatic breast cancer. In the past few years, a number of other angiogenesis inhibitors have received FDA approval for treatment of various diseases. In 2004, an anti-VEGF aptamer (Pegaptanib, Macugen), was approved for the treatment of age-related macular degeneration. FDA also approved Erlotinib (Tarceva), a small molecule inhibitor of EGF receptor tyrosine receptor kinase, for the treatment of non-small cell lung cancer. In 2005, Endostatin (Endostar), a fragment of Collagen XVIII that inhibits metastasis and angiogenesis by downregulating multiple angiogenic factors, was approved in China for the treatment of advanced lung cancer. In the same year, Sorafenib (Nexavar), a multi-tyrosine kinase inhibitor, was approved by FDA as second-line therapy for advanced renal cancer. Lenalidomide (Revlimid), an agent with both immunomodulatory and antiangiogenic properties, was also approved by FDA for the treatment of myelodysplastic syndrome. Two anti-angiogenesis drugs were approved by FDA in 2006. Sunitinib (Sutent), a multi-tyrosine kinase inhibitor, was approved as first-line therapy for advanced renal cancer and gastrointestinal stromal tumor (GIST); and Ranibizumab (Lucentis), a fragment of the bevacizumab molecule, was approved for the treatment of age-related macular degeneration. In 2007, FDA approved mTOR inhibitor Temsirolimus (Torisel) for the treatment of advanced renal cancer, and VEGF inhibitor Sorafenib for the treatment of unresectable advanced hepatocellular carcinoma and advanced renal cancer who failed first-line therapy. Many decades of research on angiogenesis and anti-angiogenesis have finally been paid off by these FDA approved drugs that directly benefit patients and by a repertoire of candidate drugs that can be further developed into clinical use. Among them, ANG inhibitors hold particular promise owing to the essential role of ANG-mediated rRNA transcription in angiogenesis in general. ANG inhibitors will be effective in inhibiting angiogenesis induced not only by ANG but also by other angiogenic factors. Agents that inhibit ANG are thus more effective than those that target other individual angiogenic factors. Moreover, the unique property of HNSCC, such as the propensity of multiple primary tumors,¹¹⁵ high vascular nature of the tumors,¹⁷ unresectability of some primary tumors² but relatively easy accessibility to topical therapeutic agents, makes HNSCC an appropriate cancer type with which anti-ANG agents can be tested and developed into clinical therapy.

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Table 1

Angiogenic polypeptides (selected examples)

Angiogenic proteins	Endothelial mitogenicity	Endothelial mobility	Reference
Acidic fibroblast growth factor (aFGF)	+	+	29
Angiogenin (ANG)	+	+	30
Basic fibroblast growth factor (bFGF)	+	+	31
Epidermal growth factor (EGF)	+	+	32
Follistatin	+	+	33
Leptin	+	+	34
Midkine and pleiotrophin	+	+	35
Platelet-derived endothelial cell growth factor (PD-ECGF)	+	-	36
Vascular endothelial growth factor (VEGF)	+	+	37
Placental growth factor (PIGF)	+	+	38
Hepatocyte growth factor (HGF)	+	+	39
Platelet-derived growth factor (PDGF)	+	+	40
Platelet activating factor (PAF)	-	+	41
Interleukin-8 (IL8)	+	+	42
Granulocyte-colony stimulating factor (GCSF)	+	+	39
Proliferin	-	+	43
Tat protein of HIV-1	+	+	44
Insulin-like growth factor (IGF)	+	-	45
Tumor necrosis factor- α (TNF- α)	-	-	46
Transforming growth factor- β (TGF- β)	-	-	47