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## Are the data on quality of life and patient reported outcomes from clinical trials of metastatic non-small-cell lung cancer important?

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**Key words:** Quality of life; Clinical trials; Non-small cell lung cancer; Patient reported outcomes

**Core tip:** Are the data on quality of life (QOL) and patient reported outcomes (PROs) from clinical trials of metastatic non-small-cell lung cancer important? Yes, they are important if the data of PROs and QOLs questionnaires are collected appropriately with a good patient's compliance.

### Abstract

Majority of the patients with advanced non-small-cell lung cancer (NSCLC) experience two or more disease related symptoms, which may have a negative impact on their health-related quality of life (HR QOL). These patients prefer a therapy that would improve disease related symptoms, as opposed or treatment that slightly prolongs their survival without improving symptoms. The improvements of the symptoms augment the significance of improved response rates or progression free survivals. The choice of the questionnaires to evaluate patients-reported outcomes (PROs) and HRQOL benefits and methods of collecting the data and their interpretations are very important and are discussed in this manuscript. PROs and HR QOL outcomes are important in patients with advanced NSCLC only when the data are collected and analyzed correctly. Then they can be viewed as components of the total value of a treatment, providing a comprehensive picture of the benefits and risks of anticancer therapies. Enabling the patients to feel during the last months of their lives more comfortable and not be dependent on their loved ones is a very important task in the treatment of advanced NSCLC.

Hirsh V. Are the data on quality of life and patient reported outcomes from clinical trials of metastatic non-small-cell lung cancer important? *World J Clin Oncol* 2013; 4(4): 82-84 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v4/i4/82.htm> DOI: <http://dx.doi.org/10.5306/wjco.v4.i4.82>

### INTRODUCTION

Lung cancer is a leading cause of cancer death worldwide for both men and women<sup>[1]</sup>.

Majority of the patients present at the time of diagnosis with metastatic disease. Of these patients with advanced non-small-cell lung cancer (NSCLC) approximately 90% of patients experience two or more disease related symptoms such as cough, dyspnea, pain and the general symptoms of fatigue and anorexia<sup>[2]</sup>. All these symptoms may cause psychological distress and may have a negative impact on a patient's health-related quality of life (HRQOL). High degrees of psychological distress influence the emotional well-being in both patients and their families. It is not surprising that 68% of patients would prefer a therapy that would improve disease-

related symptoms without prolonging life, as opposed to treatment that slightly prolonged survival without improving symptoms<sup>[3]</sup>.

Treatment can affect a patient's well-being through both symptom control and treatment-related toxicity<sup>[4]</sup>. Therefore; treatments which can decrease tumour growth (achieve a tumour response) and at the same time be less toxic, are very important for these patients<sup>[4,5]</sup>. It is important for patients to preserve their independence and not be dependent on their loved ones, becoming a burden at the end for their lives<sup>[6-8]</sup>.

The response to treatment can have an effect on disease-related symptoms and some studies suggest a link between tumour response and symptoms such as cough, dyspnea, chest pain and also systemic symptoms such as fever, anorexia and weight loss<sup>[9-11]</sup>. The improvements of these symptoms further augment the significance of improved response rates or progression free survivals (PFS). Median overall survival for most of the patients with metastatic NSCLC is modest, around one year; in epidermal growth factor receptor mutations positive tumors it approaches two years, thus HRQOL and patients-reported outcomes (PROs) carry high importance.

## METHODS OF COLLECTING THE DATA

Patient-reported symptoms (outcomes) and HRQOL benefits are usually assessed using the self-administered cancer-specific European Organisation for Research and Treatment of cancer (EORTC) questionnaires QLQ C30<sup>[12]</sup> the lung cancer-specific EORTC QLQ-LC 13<sup>[13]</sup> and the Euro QOL EQ-5D<sup>[14]</sup> questionnaire or FACT-L<sup>[15]</sup> (functional assessment of cancer treatment in lung cancer) questionnaire. The QLQ-C30 questionnaire consists of five functional scales (physical, role, cognitive, emotional and social functioning), three symptom scales (fatigue, pain, nausea/vomiting), a global health status/QOL scale and single items, *i.e.*, dyspnea, loss of appetite, constipation, diarrhea, sleep disturbance and financial impact. The QLQ LC 13 questionnaire incorporates one multi-item scale to assess dyspnea and a series of single items assessing cough, pain, sore mouth, dysphagia, peripheral neuropathy, alopecia and use of pain medication. For each scale/item, a linear transformation was applied to standardize the raw score for a range from 0-100, with 100 representing best possible function/QOL for functional scales, and highest burden of symptoms for symptom scales and symptom items.

A 10-point change in an item or domain is perceived to be clinically meaningful<sup>[16]</sup>. The percentage of patients who are classified as improved ( $\geq 10$ -point increase for functioning scales and  $\geq 10$ -point reduction for symptom domains or items from baseline scores) with respect to each of the questionnaires is examined<sup>[16]</sup>. In addition, time to deterioration of an item/domain score is defined as the item from randomization to the first appearance of a score that is 10-points or more lower or higher than the baseline score ( $\geq 10$ -point reduction for function-

ing scales and  $\geq 10$ -point increase for symptom scales or items). The EQ-5D is a disease-generic questionnaire that comprises the EQ-5D and EQ-visual analogue scale (VAS). The EQ-5D measures five dimensions of health (mobility, self-care, usual activities, pain/discomfort and anxiety/depression). Each dimension comprises three levels (no problems, some/moderate problems and extreme problems). Utility scores range from 0-1 and were calculated from the five EQ-5D items scores using the United Kingdom preference weights<sup>[17]</sup>. The EQ-VAS records the patient's self-rated health status on a vertical, graduated (0-100) VAS. Functional Assessment of Cancer Therapy-Lung (FACT-L) questionnaire (version 4) comprises 36 items across 5 domains/categories, *i.e.*, physical, social, family, emotional and functional well-being. Lung cancer subscale consists of *i.e.*, symptoms, cognitive function and regret of smoking. Scores range from 0 (not at all) to 4 (very much)<sup>[15]</sup>.

Each protocol specifies schedule for questionnaires to be completed, *i.e.*, at baseline, every 2-4 wk, at the end of treatment visit and during the first follow-up visit. The use of concomitant medications has to be assessed at the baseline and during the trial, especially the analgesic use, anti-anxiety, depression medication, O<sub>2</sub> use, *etc.*

## RESULTS AND THEIR INTERPRETATION

In order to obtain reliable results, patients have to answer the questionnaires prior to meeting their physicians and finding out results of their tests (scans). Help with the questionnaires should be available by knowledgeable staff in the clinic/hospital. The questionnaire has to be filled out by the patients themselves, not by other family member. A supervision to ensure objectivity is important.

The attention has to be paid to baseline scores. In randomized trials, are they well balanced? Are they low (= low burden of symptoms) or high (= high burden of symptoms)? If the baseline scores are low, the percentage of patients with improved symptoms on certain anti-cancer treatment might be difficult to find. On the other hand, time to symptom deterioration (= delay of deterioration) might be of high importance. Also the longitudinal analysis looking at symptoms and HR QOL over time, at different visit intervals might be informative.

The compliance of the patients with the questionnaires should always be mentioned. One would like the compliance to remain through the study at  $\geq 80\%$ , in order to be able to analyse and interpret the results appropriately. In case of EORTC questionnaires, both EORTC QLQ LC 13 and QLQ C30 should be analysed to obtain a complete picture of not only lung cancer related symptoms, but also of symptoms related to cancer treatment toxicities.

The patient's symptoms are treated, especially the last months of life, by analgesics, cough suppressants, O<sub>2</sub>, antidepressants, appetite stimulating agents and other supportive measures, which in final analysis, have to be incorporated. Other factors, such as performance status



(improving or deteriorating), weight loss and need for special emotional counselling are of great value in understanding the total value of lung cancer treatments.

## CONCLUSION

In addition to efficacy and safety endpoints, PROs and HRQOL outcomes are important in patients in advanced NSCLC, when the data are collected and analysed correctly. They should be viewed as components of the total value of a treatment. They should provide, together with the other concern endpoints, a comprehensive picture of the benefits and risks of anticancer therapies. This position has been taken by Food and Drug Administration, (2003) and European Medicine Agency<sup>[18,19]</sup>.

To collect and analyse the PROs and HRQOL data with high quality, completeness and an excellent patient's compliance, a dedicated personnel is required. The process is time-consuming, it has to be a team work of knowledgeable, devoted workers, who are ready to participate in clinical trials and thus deliver reliable results of PROs and HR QOL questionnaires. Obtaining not only prolonged PFS, but enabling patients to feel during the last months of their lives more comfortable and independent, is a very important task in the treatment of advanced NSCLC.

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## Squamous cell carcinoma of the skin: Emerging need for novel biomarkers

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### Abstract

The incidence of non-melanoma skin cancers (NMSC) is rising worldwide resulting in demand for clinically useful prognostic biomarkers for these malignant tumors, especially for invasive and metastatic cutaneous squamous cell carcinoma (cSCC). Important risk factors for the development and progression of cSCC include ultraviolet radiation, chronic skin ulcers and immunosuppression. Due to the role of cumulative long-term sun exposure, cSCC is usually a disease of the elderly, but the incidence is also growing in younger individuals due to increased recreational exposure to sunlight. Although clinical diagnosis of cSCC is usually easy and treatment with surgical excision curable, it is responsible for the majority of NMSC related deaths. Clinicians treating skin cancer patients are aware that certain cSCCs grow rapidly and metastasize, but the underlying molecular mechanisms responsible for the aggressive progression of a subpopulation of cSCCs remain incompletely understood. Recently, new molecular markers for progres-

sion of cSCC have been identified.

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**Key words:** Squamous cell carcinoma; Skin cancer; Biomarker; Matrix metalloproteinase; Serpin

**Core tip:** Several molecular markers for progression of cutaneous squamous cell carcinoma (cSCC) have been identified, but a clinically useful panel of biomarkers is still not available. Further studies are required to determine whether prognostic cSCC biomarker panel can be incorporated into clinical practice. In the meantime, while waiting for novel diagnostic and prognostic tools, clinicians must actively advocate public awareness on skin protection against excessive sun exposure in order to lower the increasing incidence of cSCC.

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### INTRODUCTION

The incidence of cutaneous squamous cell carcinoma (cSCC) is increasing worldwide<sup>[1,2]</sup>. SCC of the skin is responsible for the majority of non-melanoma skin cancer (NMSC) deaths, as invasive cSCC displays a potential for recurrence and metastasis<sup>[3]</sup>. At present, cSCC is primarily a disease of the elderly, but the incidence is also increasing in younger individuals due to excessive recreational exposure to sunlight<sup>[4]</sup>. The rising incidence of cSCC is also a reason for increased demand for medical care related to skin cancer which is estimated to grow 5% annually in the Central Europe<sup>[5]</sup>. Public awareness of skin

**Table 1 Risk factors for the development of squamous cell carcinoma of the skin**

Exposure to ultraviolet (UV) radiation (UVA, UVB)
Therapy with methoxalen and UVA
Fair skin type
Ionizing radiation
Genodermatosis (albinism, xeroderma pigmentosum, epidermolysis bullosa)
Chronic inflammation of skin (lupus erythematosus, epidermolysis bullosa)
Chronically injured skin with ulcers (burn scars, leg ulcers, epidermolysis bullosa)
Human papilloma virus infection
Exposure to chemical carcinogens
Immunosuppression
Immunosuppressive medications
Organ transplantation
Osteomyelitis
Sinus tracts
Precursor lesions (actinic and arsenical keratoses)
<i>In situ</i> squamous cell carcinoma (Bowen's disease and Erythroplasia Queyrat)
Tobacco smoking
Leukemia and lymphoma

Originated from Madan *et al*<sup>[2]</sup>, with permission.

cancer as a potentially lethal disease should therefore be fostered, and the growing economical burden of rising skin cancer incidence to the societies should be taken into consideration in healthcare planning<sup>[6,7]</sup>. In addition to promoting avoidance of excessive sun-exposure, early lesion skin biopsy and treatment of premalignant lesions are essential in prevention of cSCC<sup>[8]</sup>. To ensure early diagnosis, dermatologic expertise is obviously needed, and this should be taken into account in planning of medical education<sup>[9]</sup>.

The treatment of choice for primary cSCC is surgical excision, whereas Mohs micrographic surgery is recommended for high-risk cases<sup>[1,10]</sup>. Unfortunately, excision is not always curative, as cSCCs have an overall 5-year recurrence rate of approximately 5%<sup>[11]</sup>. Here, the challenge faced by clinicians is to identify the high-risk cases before the recurrence and metastasis of the tumor. There is an obvious demand for predictive molecular markers that could be used at the time of excision of the primary tumor for evaluation of the risk of recurrence and metastasis. Furthermore, if dissemination of the tumor has already taken place, novel targeted therapies are needed.

In this editorial, we discuss the molecular pathways involved in the development of invasive cSCC and the risk factors for cSCC progression, recurrence and metastasis. Finally, recent progress in the search for cSCC progression biomarkers will be discussed.

## MORBIDITY AND ECONOMICAL BURDEN OF cSCC

NMSC, including cSCC, are among the most commonly diagnosed cancers in Europe, United States and Aus-

tralia<sup>[12-14]</sup>. Moreover, the incidence of cSCC is steadily increasing worldwide, especially among the white population living in the proximity of the equator<sup>[1]</sup>. Interestingly, the incidence of cSCC is also rising in the less sun exposed regions of the globe, as approximately 4% annual increase in the incidence has been registered in Finland during the past decades<sup>[15]</sup>. The growing number of new cSCC cases, as well higher incidence of recurrent tumors makes cSCC one of the costliest cancers in many countries<sup>[7,16,17]</sup>. It is conceivable, that the cost of NMSC to the societies will continue to grow due to the extension of individual lifespan and aging of the population.

One of the main reasons for the rising incidence of cSCC is popularity of recreational sun-exposure despite growing public awareness of the harmful effects of solar ultraviolet (UV)-light<sup>[4]</sup>. On the other hand, the number of individuals receiving long-term immunosuppressive medication after organ transplantation has increased. As organ transplant recipients have a 65-fold higher risk of developing cSCC, regular follow-up of these individuals is mandatory and early diagnosis of cSCC, preferably at pre-malignant stage, is essential<sup>[18]</sup>. Even if the skin malignancies are diagnosed at premalignant stage, the treatment may be costly due to the field cancerization of the skin and the relatively high-cost of topical therapies available. Interestingly, kidney transplant recipients with a history of cSCC also have a higher risk for internal malignancies<sup>[19]</sup>. It has been proposed that switching the immunosuppressive medication from calcineurin inhibitors to inhibitors of the mammalian target of rapamycin, such as sirolimus could have an antitumoral effect among kidney-transplant recipients with previous cSCC<sup>[20]</sup> but this observation has been challenged<sup>[21]</sup>.

For dermatologists, diagnosis of cSCC and its precursor, actinic keratosis and cSCC *in situ* (Bowen's disease), is often easy by visual inspection<sup>[22]</sup>. However, clinical diagnosis may sometimes be challenging, especially in patients suffering from severe generalized form of recessive dystrophic epidermolysis bullosa (gs-RDEB), with multiple chronic ulcers, mimicking malignant lesions<sup>[23]</sup>.

## RISK FACTORS FOR DEVELOPMENT OF cSCC

Sunlight has a vital role on the Earth as the primary source of energy, but it is also the primary cause of skin cancer<sup>[24]</sup>. Theoretically, UV-radiation as the major risk factor for cSCC could be avoided, but avoiding sun exposure is a challenge in daily life<sup>[4]</sup>. UVB (wavelength 280-320 nm) radiation can damage both DNA and RNA directly leading to the generation of mutagenic photo-products such as pyrimidine-pyrimidine adducts and cyclopuridine dimers<sup>[25]</sup>. UVA (wavelength 320-400 nm) radiation damages DNA indirectly *via* a photo-oxidative-stress-mediated mechanism which results in DNA double-strand breaks<sup>[26,27]</sup>. As both UVB and UVA are carcinogenic, the sunscreen used should block both UVB and UVA rays<sup>[28]</sup>. The risk factors for the development of

**Table 2 Risk factors for recurrence and metastasis of cutaneous squamous cell carcinoma**

Variable	Approximate relative risk <sup>1</sup>	
	Recurrence	Metastasis
Rapid tumor growth	-	-
Tumor size > 2 cm	2	2
Tumor location (lip/ear)	2	3
Immunosuppression	-	2
Previous radiotherapy	-	-
Previously treated cSCC	3	4
RDEB -associated cSCC	-	-
Tumor depth > 4 mm	2	5
Poor differentiation	2	3
Acantholytic features	-	-
Spindle-cell features	-	-
Perineural invasion	5	5

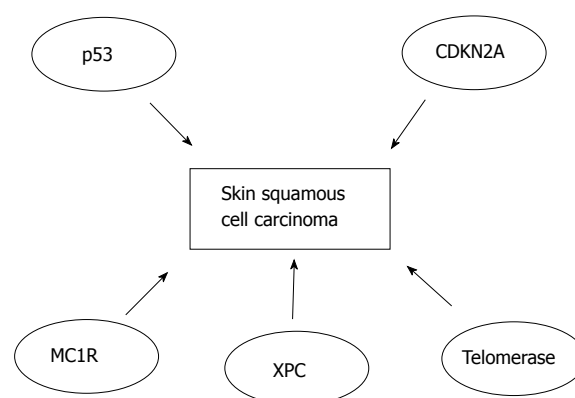
Originated from Alam *et al.*<sup>[1]</sup>, with permission. <sup>1</sup>A relative risk of 1 is defined as the likelihood of recurrence or metastasis of a small primary cutaneous squamous cell carcinoma (cSCC). Dashes indicate an association with increased risk, but of which there are insufficient data to estimate the relative risk. RDEB: Recessive dystrophic epidermolysis bullosa.

cSCC are summarized in Table 1.

## MOLECULAR PATHWAYS INVOLVED IN THE DEVELOPMENT OF cSCC

Major pathways involved in the pathogenesis of SCC of the skin are shown in Figure 1. Early inactivation of both alleles for tumor protein 53 gene (*TP53*) has an important role in the development of cSCC<sup>[29,30]</sup>. *TP53* mutations are observed in roughly 90% of all cSCCs and these mutations occur mainly due to UV radiation. Following inactivation of both *TP53* alleles, a marked expansion in simple mutations takes place making cSCCs the human cancer with highest mutation rate known<sup>[31]</sup>. Subsequently, epidermal keratinocytes undergo malignant transformation and clonal expansion will occur which is clinically manifested as the development of early *in situ* SCC, actinic keratosis<sup>[30,32]</sup>. In patients with xeroderma pigmentosum, mutations in xeroderma pigmentosum complementation group C, that lead to failure to repair DNA, are the key event of cSCC development<sup>[33]</sup>. Melanocortin-1 receptor variants are associated with fair skin, red hair and increased risk of developing melanoma, and they are also an independent risk factor for development of SCC of the skin<sup>[34]</sup>. In addition, telomerase activity may be elevated in cSCC leading to immortalization of tumor cells<sup>[35]</sup>. Moreover, inactivation of cyclin-dependent kinase inhibitor 2A locus has been detected in SCC of the skin<sup>[36]</sup>. Moreover, loss of function mutations of *NOTCH-1* and *NOTCH-2* genes have been noted in 75% of cSCCs emphasizing the importance of *NOTCH* genes as tumor suppressors in these epithelial malignancies<sup>[37]</sup>.

Chronic skin exposure to UV light results in DNA damage and mutations in the genes mentioned above leading to malignant transformation of keratinocytes. Moreover, UV light can promote cSCC tumorigenesis



**Figure 1 Pathways involved in the pathogenesis of cutaneous squamous cell carcinoma.** Originated from Madan *et al.*<sup>[2]</sup>, with permission. p53: Tumor protein 53; CDKN2A: Cyclin-dependent kinase inhibitor 2A; MC1R: Melanocortin 1 receptor; XPC: Xeroderma pigmentosum complementation group C.

and progression also *via* other mechanisms, such as immunosuppression and inhibition of macrophage migration<sup>[28,38]</sup>.

## RECURRENCE AND METASTASIS OF cSCC

SCC of the skin is often relatively rapidly growing, locally invasive malignant tumor that has potential for recurrence and metastasis, with an overall 5-year recurrence rate of 8% and a 5-year rate of metastasis of approximately 5%<sup>[11]</sup>. The location and the size of the primary tumor are relevant in the assessment of the risk of recurrence and metastasis of a given tumor. Typical high-risk anatomical areas are lips and ears, as cSCC in these areas recur and metastasize at a rate of 10% to 25%. The relatively low general risk of recurrence and metastatic spread is markedly higher if the primary lesion is large, as tumors with a diameter > 2 cm show a recurrence rate of 15% and metastasis rate of 30%<sup>[11]</sup>. In certain cSCC subtypes, such as as in tumors arising in chronic ulcers or chronically injured skin, the risk of metastasis may be as high as 40%<sup>[39,40]</sup>. In addition, certain histological features of cSCC are known to be related to poor prognosis<sup>[1,41]</sup>. These features are shown in the Table 2.

Although these clinical and histological risk factors have been established, clinicians treating patients with cSCC still do not have access to molecular tools to assess the risk of recurrence and metastasis in a given patient. Thus, novel molecular biomarkers would be of great value in the risk assessment.

## SEARCH FOR NOVEL BIOMARKERS FOR cSCC PROGRESSION

In our own studies, we have searched for novel biomarkers for progression of cSCC. We have utilized a diverse range of research methods to identify relevant candidate genes and tumor proteins. The first step has been genome-wide expression profile analysis of cSCC cell



lines *vs* normal human epidermal keratinocytes<sup>[42]</sup>. Secondly, we have validated the expression profiling data at the mRNA level with quantitative real-time polymerase chain reaction<sup>[43]</sup>. Then, we have confirmed the findings at the protein level with Western blotting<sup>[44]</sup>. As cultured tumor cells represent only a selected portion of a given tumor, we have collected a large panel of *in vivo* tissue samples containing normal human skin, actinic keratoses, cSCCs *in situ* (Bowen's diseases) and cSCCs<sup>[42-44]</sup>. These formalin fixed, paraffin embedded samples were used for immunohistochemical studies as tissue microarrays<sup>[42-44]</sup>. In addition, we have used chemically induced mouse skin carcinogenesis model for validation of our human data<sup>[42]</sup>.

## ROLE OF MATRIX

### METALLOPROTEINASE-7 IN cSCC PROGRESSION

Matrix metalloproteinases (MMP) contribute to the homeostasis of a variety of tissues and participate in many physiological processes, such as proteolysis of extracellular matrix in skin<sup>[45]</sup>. Upregulation of MMP expression has been seen in many different types of cancers, including cSCC<sup>[46,47]</sup>.

In recent studies, we showed that the expression and production of MMP-7 is specifically elevated in cSCCs<sup>[43,44]</sup>. Interestingly, MMP-7 expression was even more abundant in the gs-RDEB-associated cSCCs representing an aggressive subtype of SCC of the skin<sup>[43]</sup>. Immunohistochemical studies revealed elevated MMP-7 expression especially in the invasive edge of the cSCC tumors<sup>[43]</sup>.

Furthermore, we studied the mechanistic role of MMP-7 in cSCC and noted that MMP-7 activates heparin binding epidermal growth factor-like growth factor (HB-EGF) in cSCC cells<sup>[44]</sup>. In functional studies, proliferation of cSCC cells was suppressed when the activation of HB-EGF by MMP-7 was inhibited<sup>[44]</sup>. These findings provide mechanistic evidence for proposed therapeutic effect of epidermal growth factor receptor antagonists in treatment of advanced cSCCs.

### SERINE PEPTIDASE INHIBITOR CLADE A MEMBER 1 AS A NOVEL BIOMARKER FOR PROGRESSION OF cSCC

Serine peptidase inhibitors (Serpins) constitute the largest and most broadly distributed superfamily of peptidase inhibitors described in humans<sup>[48,49]</sup>. We studied the gene expression levels of entire serpin family in cSCC cell lines *vs* normal keratinocytes and found that expression of SerpinA1, also known as 1-antitrypsin, was markedly elevated<sup>[42]</sup>. Furthermore, elevated SerpinA1 expression correlated with the tumorigenic potential of transformed keratinocytes<sup>[42]</sup>. Moreover, SerpinA1 expression in SCC tumor cells *in vivo* correlated with tumor progression<sup>[42]</sup>.

Furthermore, SerpinA1 expression was clearly more abundant in gs-RDEB-associated cSCCs representing an aggressive subtype of cSCC<sup>[42]</sup>. To further verify the role of SerpinA1 in the progression of cSCC, we used chemically induced mouse skin carcinogenesis model that showed correlation with SerpinA1 expression and progression of mouse skin SCC<sup>[42]</sup>. Our findings clearly demonstrate that SerpinA1 may serve as a useful biomarker for progression of cSCC.

## CONCLUSION

Although patients with cSCC in general do not have as poor prognosis as those with melanoma, the impact of cSCC to the quality of life of the patients, as well as to the societies in general will be greater in the near future due to the increased incidence of this malignant tumor and the longer life-span of the population. To improve the accuracy of diagnosis and the assessment of individual prognosis, there is a demand for novel biomarkers for progression of cSCC. We have identified potential biomarkers for this purpose, but further research is required to validate their feasibility in clinical practice. As cSCC is not a uniform disease but rather a heterogenous group of tumors, we assume that a single biomarker probably will not be sufficient, but a panel of biomarkers is needed for making clinical decisions. Finally, the power of preventive measures against skin cancer should not be underestimated. For this purpose, physicians, together with other healthcare professionals, must actively promote public awareness of skin protection against excessive sun exposure.

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## Monitoring adenoviral based gene delivery in rat glioma by molecular imaging

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### Abstract

**AIM:** To determine whether endothelial progenitor cells (EPCs) can be used as delivery vehicle for adenoviral vectors and imaging probes for gene therapy in glioblastoma.

**METHODS:** To use cord blood derived EPCs as delivery vehicle for adenoviral vectors and imaging probes for glioma gene therapy, a rat model of human glioma was made by implanting U251 cells orthotopically. EPCs were transfected with an adenovirus (AD5/carrying *hNIS* gene) and labeled with iron oxide and inoculated them directly into the tumor 14 d following implantation

of U251 cells. Magnetic resonance imaging (MRI) was used to *in vivo* track the migration of EPCs in the tumor. The expression of gene products was determined by *in vivo* Tc-99m single photon emission computed tomography (SPECT). The findings were validated with immunohistochemistry (IHC).

**RESULTS:** EPCs were successfully transfected with the adenoviral vectors carrying *hNIS* which was proved by significantly ( $P < 0.05$ ) higher uptake of Tc-99m in transfected cells. Viability of EPCs following transfection and iron labeling was not altered. *In vivo* imaging showed the presence of iron positive cells and the expression of transgene (*hNIS*) product on MRI and SPECT, respectively, all over the tumors following administration of transfected and iron labeled EPCs in the tumors. IHC confirmed the distribution of EPC around the tumor away from the injection site and also showed transgene expression in the tumor. The results indicated the EPCs' ability to deliver adenoviral vectors into the glioma upon intratumor injection.

**CONCLUSION:** EPCs can be used as vehicle to deliver adenoviral vector to glioma and also act as imaging probe at the same time.

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**Key words:** Cord blood endothelial progenitor cells; Adenovirus; Human sodium iodide symporter; Single photon emission computed tomography; Magnetic resonance imaging

**Core tip:** Endothelial progenitor cells (EPCs) can be transfected with replication competent adenoviral vector carrying therapeutic/reporter gene and the transfected EPCs can be used to deliver the gene in tumor gene therapy. EPCs can be used as both imaging and therapeutic probes.

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## INTRODUCTION

Glioblastoma (GBM) is one of the aggressive primary brain tumors with survival period falls from one to three years upon treatment<sup>[1,2]</sup>. Today's available treatments (chemotherapy, radiation and surgery) have little success and alternative or combinational approaches to control gliomas are in need<sup>[3]</sup>. In alternative treatments, gene therapy has shown promise due to development of various tools such as lentivirus, adenovirus, adeno-associated virus, conditional replicating viruses and tumor specific promoters<sup>[3-5]</sup>. In a gene therapy approach, therapeutic or suicidal genes or oncolytic viruses are delivered into the tumor cells to suppress or eliminate tumor growth<sup>[3,6]</sup>. Numerous studies have shown the ability of oncolytic viruses to suppress the tumor cells and have been proven to be safe in clinical trials<sup>[4,5,7]</sup>. The efficacy of the oncolytic agents, however, may be compromised due to their limited infection efficiency, mode of delivery to the tumors, and distribution into the entire tumor area<sup>[6]</sup>. Currently, different oncolytic viral vectors are being administered directly into the tumor sites by multiple intratumor injections<sup>[4,8,9]</sup>. Intratumor injection is advantageous compared with systemic injection of a virus to control entry into the circulation and to avoid unexpected side effects<sup>[10]</sup>. Strategies for improved gene delivery are highly desirable to overcome some of these shortcomings. In this context, genetically transformed cells are being considered as delivery vehicles to deliver genes or viruses directly into different tumors<sup>[11-14]</sup>. Due to their unique property to migrate to the pathological lesions, stem cells are considered a unique choice to be the delivery vehicle for therapeutic genes to the tumors, especially for glioma<sup>[15-17]</sup>. Endothelial progenitor cells (EPCs) are a subpopulation of pluripotent hematopoietic stem cells (HSC), which show active migration and incorporation into the neovasculatures of glioma when administered locally or systemically<sup>[18-21]</sup>. Based on the characteristics of EPCs, it is possible to use these cells as vehicles for the delivery of therapeutic genes to gliomas (using viral vectors)<sup>[14]</sup>. In addition, EPCs can be collected from a patient's own peripheral blood and bone marrow, which in turn eliminate the possibility of immune response<sup>[22]</sup>.

Locally administered transfected EPCs can act as delivery vehicles for genes, viral vectors or both. For example, transgenic EPCs can carry either a therapeutic gene, such as growth inhibitory or inflammatory factors, or a suicidal gene, such as HSV-tk for antiviral drugs or human sodium iodide symporter (hNIS) for I-131 (radioiodine) or both<sup>[7,23,24]</sup>. The delivery of genes from trans-

fected EPCs into adjacent tumor cells can be achieved by using replication competent viral vectors that will enable the virus to grow inside the EPCs, shed, and transfect the adjacent tumor and neovasculature cells<sup>[25]</sup>. Upon effective infection of the tumor and adjacent neovasculature cells, anti-tumor treatment can be started, especially if suicidal genes are used<sup>[5,7,23,24]</sup>. Local administration of virus-transfected EPCs will have advantages over the direct injection of viral particles to the tumors for the following reasons: (1) EPCs will not allow quick release of viral vectors to the circulation through blood brain barrier; (2) local administration will prevent accidental transfection of cells in non-target organs or tissues; and (3) locally administered EPCs may migrate not only to the periphery but also to the center of the tumor and incorporate into neovasculatures<sup>[14,25-27]</sup>. Therefore, there is a higher chance for the extensive transfection of tumor cells during the migration of transfected EPCs. That would enable the subsequent anti-tumor treatment (by targeting suicidal gene for example) to be more effective<sup>[7,23,24]</sup>.

Reporter gene systems have been increasingly used for monitoring gene therapy in various tumor models to *in vivo* determine the delivery and expression of transgene products. hNIS is an intrinsic transmembrane glycoprotein that mediates the transport of iodides into the thyroid follicular cells<sup>[28,29]</sup>. A number of studies have demonstrated that active iodide uptake can be induced in a variety of cells<sup>[21,30,31]</sup>. This transport system also transports Tc-99m and can be imaged by gamma camera<sup>[32-34]</sup>. The expression of hNIS in the transfected cells is obviously higher than the non-transfected cells and higher signal associated with hNIS expression can be monitored with SPECT imaging. In our recent report we also showed the importance of hNIS in detecting *in vivo* gene expression<sup>[14]</sup>.

The purposes of this study was to determine: (1) whether EPCs transfected with replication competent adenoviral vectors carrying hNIS can migrate to other parts of a tumor following injection into orthotopic glioma; and (2) whether migration of EPCs and the delivery of the gene product to the tumor cells can be determined by *in vivo* imaging.

## MATERIALS AND METHODS

### CD133<sup>+</sup> cell collection and isolation

Human cord blood was collected under Henry Ford Health System Institutional Review Board (IRB) approved protocol. The cord blood was collected from placenta using published method with minor modifications<sup>[35]</sup>. In brief, placental blood directly collected into 50 mL tube contains 1 × PBS (with penicillin/streptomycin and ethylene diamine tetraacetic acid) by opening the clamps. Collected cord blood samples were maintained in ice until reaching the laboratory. Once reaching the laboratory cord blood CD133<sup>+</sup> cells were isolated using our published method<sup>[14,21,36]</sup>. The mononuclear



fraction from cord blood was separated using Ficoll density gradient centrifugation. Immunomagnetic isolation kit (Miltenyi, CA) was used to isolate the CD133<sup>+</sup> cells. Isolated CD133<sup>+</sup> cells were maintained as suspension cultures using CellGenix SCGM media (CellGenix, Germany) supplemented with 40 ng/mL of stem cell factor (SCF), 40 ng/mL of FLT3 and 10 ng/mL of thrombopoietin (TPO) (Prospec, United States).

### Production of replication competent viral vector

Replication competent adenovirus carrying *hNIS* gene was a gift from Dr. Barton, Henry Ford Hospital<sup>[7]</sup>. The full details of the construction of the adenovirus were described in the published work of Dr. Barton<sup>[7]</sup>. The adenoviral construct used in this study was Ad5-γCD/mutTK(SR39)rep-hNIS (replication-competent adenovirus)<sup>[7]</sup>. The adenoviral vector carries therapeutic genes and reporter genes. The E1 region contains therapeutic gene [yeast cytosine deaminase (γCD) and mutant herpes simplex virus thymidine kinase mutTK (SR39) fusion gene] and the E3 region contains reporter gene (*hNIS*)<sup>[7]</sup>. Transgenes were expressed under the control of human cytomegalovirus (CMV) promoter<sup>[7]</sup>. We used adenovirus under approved Institutional Recombinant DNA Biosafety Committee (IRDBC) protocol. Adenovirus was produced using published method with minor modifications<sup>[37]</sup>. To produce adenovirus, 293 cells were seeded at a density of  $1.6 \times 10^6$  per T75 flask. When they reached confluence, cells were split into three T75 flasks. When cells reached 80% confluence, the media was replaced with 5 mL of serum free Dulbecco's modified Eagle's medium (DMEM) containing adenovirus ( $2.6 \times 10^9$  viral particles per milliliter) and incubated at 37 °C, 5% CO<sub>2</sub>. After one hour incubation, 10 mL of complete media was added and incubated at 37 °C, 5% CO<sub>2</sub>. After 3 d of incubation supernatant containing adenovirus was collected and concentrated using polyethylene glycol (PEG) solution. Viral supernatants were mixed with PEG solution and incubated overnight at 4 °C. After incubation, virus was collected by centrifugation at 1500 g for 30 min. Concentration of viral particles were measured using ultraviolet-visible (UV) spectrometer. In brief, 5 μL of sample (adenovirus) was added to 495 μL of 0.1% SDS solution. After vortex optical density (OD) was measured using UV spectrometer at 260 and 280 nm wavelength. Number of viral particle in concentrated samples were calculated using following formula:  $1 \text{ OD}_{260} = 10^{12}$  viral particles per milliliter<sup>[37]</sup>.

### Transfection of EPCs

We used adenovirus in transfection experiments according to approved IRDBC protocol (Henry Ford Health system institutional recombinant DNA and biosafety committee). Adenoviral transfection of EPCs was performed according to published method with minor modifications<sup>[26]</sup>. To develop transfected EPCs, adenovirus carrying *hNIS* gene was added to sterile 1.5 mL microcentrifuge tube in 1:2000 ratio (cell:viral particle)

and incubated for 1 h at 37 °C, 5% CO<sub>2</sub>. After one hour incubation, 1-3 mL of fresh media was added and transferred to a 6-well plate and further incubated for 24 h.

### Tc-99m uptake assay

To test expression of *hNIS* gene in transfected EPCs, a Tc-99m uptake assay was performed according our published method<sup>[14]</sup>. In brief, 10 μCi of Tc-99m (Mallinckrodt, United States) was added to around  $1 \text{ to } 1.5 \times 10^6$  cells (in serum free media) and incubated at 37 °C for 30 min followed by washing twice with phosphate buffered saline (PBS) and cell associated activity in pellet was measured using gamma counter (Wizard 1420, PerkinElmer, United States). We used the same method to test the Tc-99m uptake in iron labeled transfected EPCs.

### Labeling of cells with ferumoxides

EPCs were labeled with ferumoxides using our published method<sup>[21,36]</sup>. In brief, ferumoxides (Fe) (Berlex Laboratories, United States) and protamine sulfate (Pro) were added to the cell suspension followed by 15 min incubation at 37 °C, 5% CO<sub>2</sub>. Upon incubation, complete stem cell media was added and incubated for 4 h followed by washing with PBS. Finally cells were resuspended in serum free media at the desired concentration for injection.

For cell viability, 100 μL cell suspension were mixed with trypan blue dye and observed under a microscope to determine cell viability. There were three types of cell preparations, which were injected in three separate groups of tumor bearing animals: (1) non-transfected non-FePro labeled (control cells); (2) transfected, FePro labeled; and (3) transfected, non-FePro labeled.

### Animal model

Animal experiments in this study was approved by animal care and user committee at Henry Ford Health System. Human glioma cells (U-251, gift from Dr. Steve Brown, HFHS) were cultured with DMEM supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (100 μg/mL). Upon reaching confluent, cells were collected and made a cell suspension ( $4 \times 10^5$  cells/5 μL) in serum free media before implanting in rat brain. Athymic nude rats 6-8 wk of age and 150-170 g of weight (Charles River Laboratory, Inc.) were used for the implantation<sup>[14,21,38]</sup>. Firstly, animals were anesthetized by intraperitoneal injection using ketamine/xylazine mixture (100 mg/kg ketamine, 10 mg/kg xylazine) and tumor cells were implanted according to our published methods<sup>[14,21,38]</sup>. There were at least 3 animals for each condition.

### Intratumor injection of transfected EPCs

Intratumor injection was performed according to published method with modifications<sup>[39]</sup>. After 14 d of post implantation of U251 cells in the rat brain, animals were anesthetized and their skulls were exposed. Using a dental drill a hole was made at 3 mm to the right and



1 mm anterior to the bregma, exactly at the site of tumor implantation, and a 10  $\mu$ L micro-syringe fitted with 26 s gauge-needle loaded with EPCs (around one or two million) in 5  $\mu$ L was lowered to the depth of 4 mm, then raised to the depth of 3 mm. Either transfected labeled, transfected non-labeled EPCs or control EPCs were injected stepwise at a rate of 0.5  $\mu$ L/30 s until the entire volume had been injected and syringe was withdrawn at one millimeter per minute. Bone wax was used to seal the drilled hole and finally the overlying skin was sutured.

### Magnetic resonance imaging

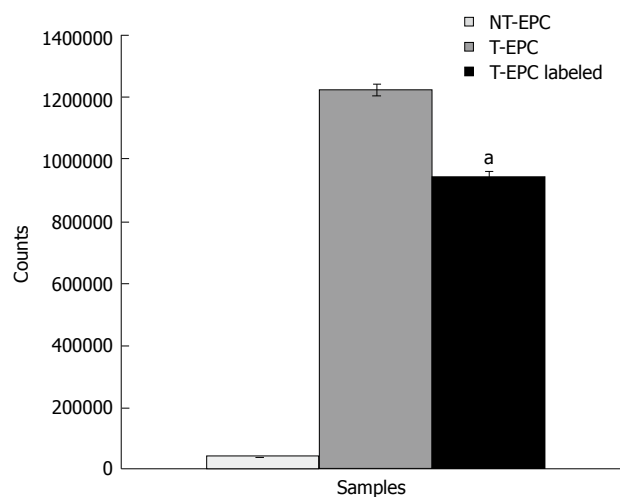
Magnetic resonance imaging (MRI) images were obtained using a 3.0 Tesla clinical system (Signa Excite, GE health) using 50 mm diameter small animal imaging coil (Litzcage small animal imaging system, Doty Scientific Inc, Columbia, SC) according our published method<sup>[14]</sup>. Rats were anesthetized with 1.5%-2.0% isoflurane in oxygen and secured in a small animal imaging coil. MRI images were acquired before and 7 d post intratumor injection of EPCs carrying adenoviral vectors. Images were obtained with three dimensional (3D) isotropic Fast Imaging Employing Steady sTate Acquisition with parameters repetition time = 11.4 ms, echo time = 5.6 ms, using a 200  $\times$  200 matrix, field of view = 60 mm and number of excitation = 2, effective slice thickness was 0.3 mm<sup>[14]</sup>.

### SPECT imaging

SPECT images were acquired based on our published method<sup>[14]</sup>. In brief, animals were anesthetized using ketamine/xylazine (100/10 mg/kg) and 1mCi of Tc-99m was injected through tail vein. After one hour of tail vein injection animals were subjected for SPECT imaging. Ketamine/xylazine (100/10 mg/kg) was used to achieve continuous state of anesthesia during 1 imaging period. SPECT was acquired with a dedicated PRISM3000 gamma camera fitted with multi-pin-hole rat collimators, 360 degree rotation with 36 degree increments, 180 s per projection, using 256  $\times$  256 matrices, with a field of view of 4 cm  $\times$  6 cm<sup>[14]</sup>. We scanned the animals for 30 min to acquire SPECT images on the tumor area<sup>[14]</sup>.

### Histological analysis

Animals were euthanized and whole brain samples were collected as described in our previous publications<sup>[14,21,38]</sup>. For histological analysis, brain samples were fixed and processed for the frozen sections. The sections were stained with Prussian blue for the detection of iron labeled cells<sup>[14]</sup>. For the detection of transgene expression, sections were stained with anti-hNIS antibody (Genetex, TX, United States). For further analysis, we used anti-vWF antibody for the detection of endothelial cells and anti-EGFR (epidermal growth factor receptor) antibody for the detection of tumor cells. Some sections were double stained to determine double expression of hNIS/vWF (hNIS expression in administered EPCs) or hNIS/EGFR (hNIS expression in surround tumor cells)<sup>[14]</sup>.



**Figure 1 Transgene expression study using Tc-99m uptake assay.** Transfected and control endothelial progenitor cells (EPCs) were subjected to Tc-99m uptake assay to determine the transgene expression. Tc-99m uptake assay was performed with three type of conditions: (1) Non transgenic, non-labeled (without iron) EPC (control); (2) transgenic, non-labeled EPC; (3) transgenic and labeled (with iron) EPCs. Transfected EPCs showed higher Tc-99m uptake compared to control non transfected EPCs, which clearly indicates the functional expression of transgene. Data was indicated with Mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  vs control cells.

### Statistical analysis

All data are expressed as mean  $\pm$  SD. A  $P$  value of  $< 0.05$  was considered significant.

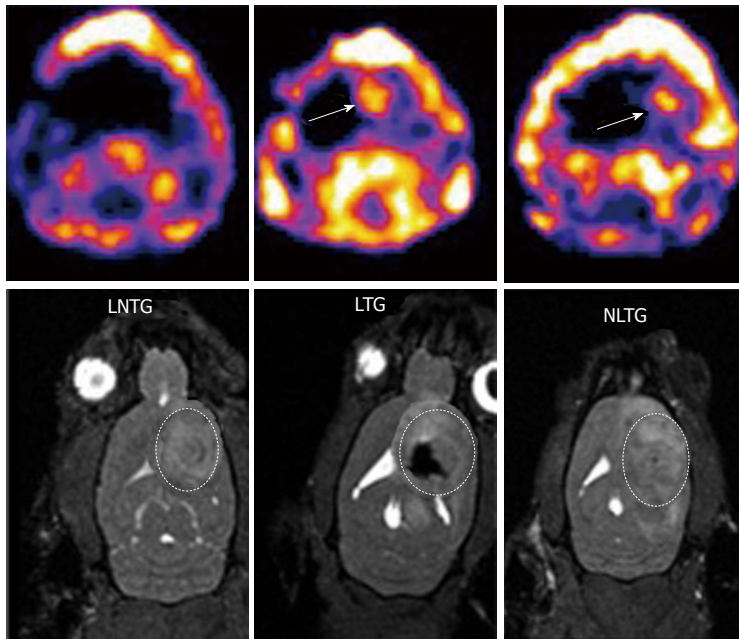
## RESULTS

### Reporter gene (hNIS) expression, viability and proliferation

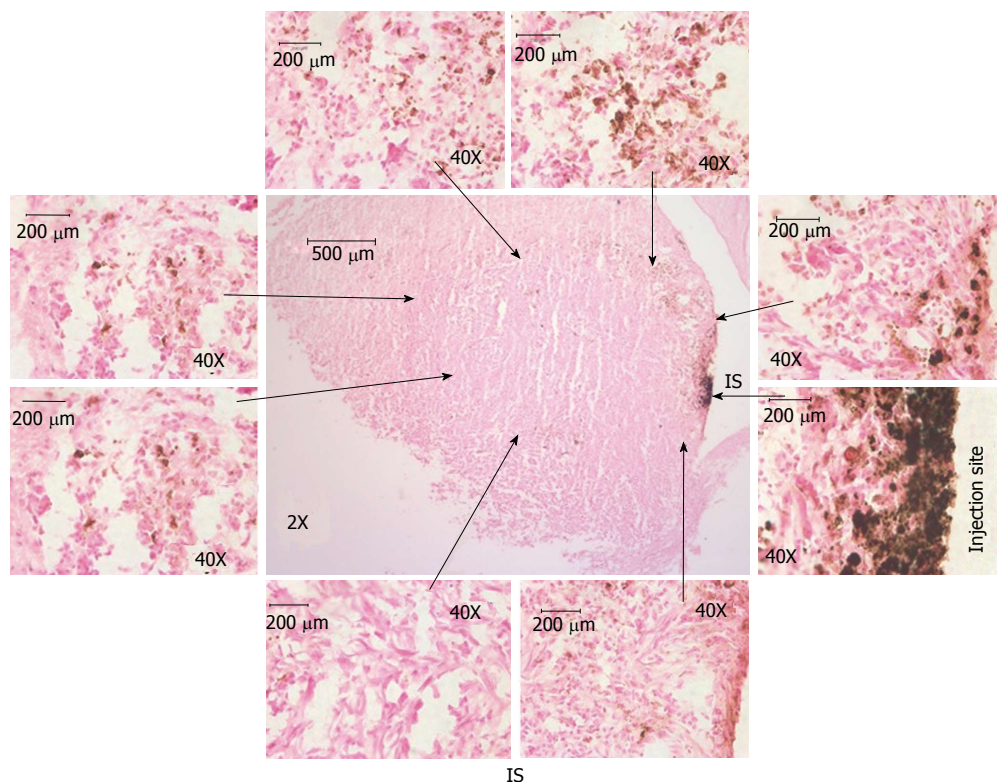
EPCs were transfected with adenovirus to study the ability of EPCs as vehicle to deliver the adenovirus into glioma. Firstly, we optimized the transfection efficiency without compromising the cell viability, which is important to determine the cell to viral dose. We studied the viral dose versus cell viability by transfecting cells with different doses of viral particles. We determined cell to viral ratio (1:2000) was optimal dose. We further studied the expression of transgene in EPCs using Tc-99m uptake assay. Figure 1 shows Tc-99m uptake was significantly higher in transfected EPCs as well as in FePro labeled transfected EPCs compared to the control EPCs. These results indicate the functional expression of hNIS gene in the EPCs.

### MRI and SPECT imaging

To study whether EPCs can deliver transgene to glioma, we injected transfected FePro labeled EPCs directly (intratumor) into glioma. Animals underwent MRI before and seven days after intratumor injection of EPCs. Figure 2 shows animals that received FePro labeled EPCs into the tumor showed low signal intensity on MRI inside the tumor. All animals that received either labeled or non-labeled transfected EPCs showed accumulation of Tc-99m in the tumor. On the other hand, animals that received non-labeled non-transduced EPCs (control) showed neither low signal intensity nor Tc-99m activity



**Figure 2** Magnetic resonance imaging and single photon emission computed tomography images for tracking of intratumor injected endothelial progenitor cells and transgene expression. Expression of transgene (*hNIS*) was detected by single photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI) was used to detect the cell migration in the tumor. Upper panels (SPECT images): Left: Animals received non transgenic, non-labeled endothelial progenitor cells (EPCs) (control); Middle: Animals received transgenic EPCs labeled with iron; Right: Animals received non labeled transgenic EPCs. Corresponding MRI images are shown in the lower panels. Animals received labeled transgenic EPC or non-labeled transgenic EPC showed higher activities of Tc-99m in the tumors compared to control animals that received non-transgenic EPCs. MRI images clearly indicate the presence of iron labeled transgenic EPCs (lower panel, middle).



**Figure 3** Histological analysis of intratumor injection and migration of cells. Animal brain sections were stained with Prussian blue and observed under light microscope. Center image (magnification  $\times 2$ ) shows the iron positive cells at injection site and migration around periphery and inside the tumor. Images  $\times 2$  shows the whole tumor area and injection sites and images  $\times 40$  shows iron labeled cell at injection site and around the tumor. Images  $\times 40$  were linked to images  $\times 2$  with arrow to show the region  $\times$  is 40 near to the arrows (not exact match). These images indicate the migration of transgenic endothelial progenitor cells (EPCs) to periphery of the tumor from the injection site. Some of the EPCs were also migrated to the center of the tumor from the injection site. IS: Injection site.

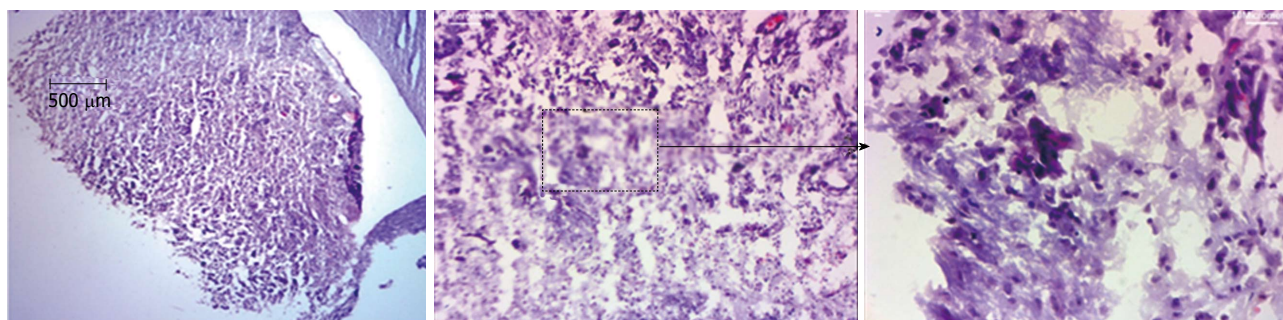
in the tumor (Figure 2).

### Histological and immunological analysis

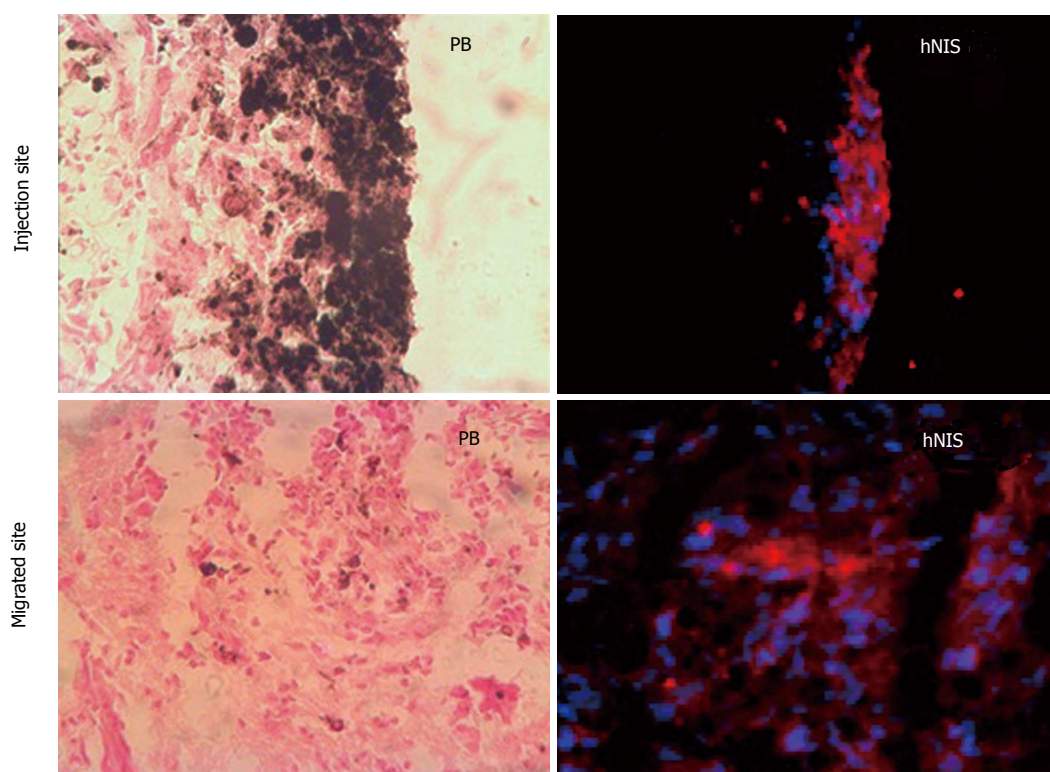
Migration of intratumor injected iron labeled EPCs in and around the tumor was detected using prussian blue staining (Figure 3). These results indicate that the EPCs are migrating from the injection sites to the pe-

riphery as well as center of the tumors. The sections also stained with HE to observe the necrotic cells, we observed necrotic cells at periphery and central regions of the tumors (Figure 4). Immunohistochemistry staining revealed the expression of transgene (*hNIS*) at the injection site and at distal areas where injected EPCs migrated (Figure 5). Double labeling of sections with





**Figure 4 Hematoxylin and eosin staining of brain sections:** Animal brain sections were stained with hematoxylin and eosin, In images  $\times 2$  show whole tumor area and images  $\times 10$  and images  $\times 40$  showing some the necrotic cells at injection site as well as at others regions of the tumor.



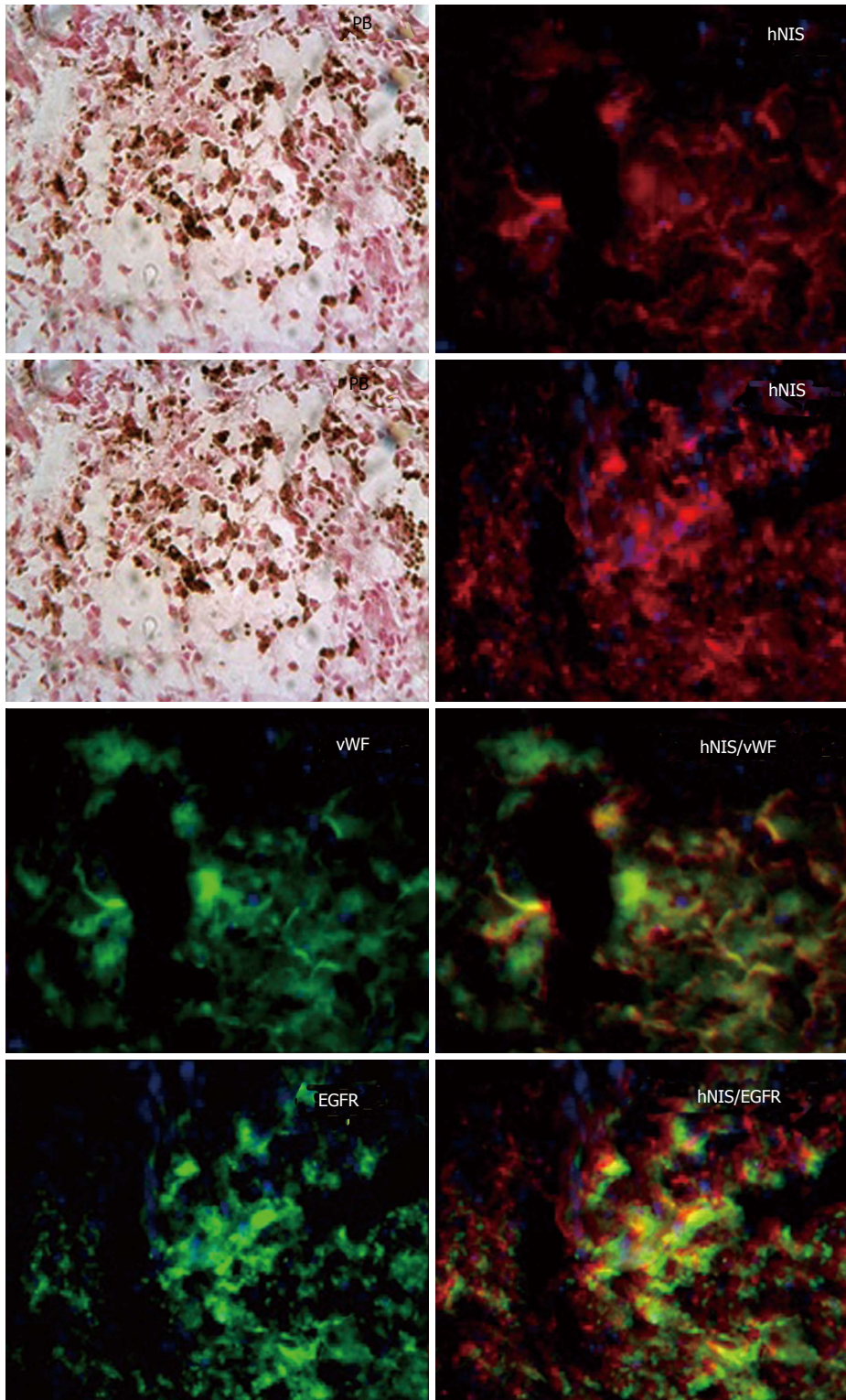
**Figure 5 Immunohistochemical analysis of transgene expression at the site of injection and migrated areas.** Red fluorescence (hNIS positive) is observed at site of injection of transgenic iron labeled endothelial progenitor cells (EPCs) and areas where injected EPCs migrated.

vWF and hNIS showed that endothelial cells migrated and expressed the transgene (Figure 6). To find out whether adenovirus also transfected the adjacent tumor cells, sections were also double stained for EGFR and hNIS. EGFR is highly expressed in glioma cells and can be used as a marker to detect the tumor cells. In double labeling, some of the hNIS positive cells also stained for the EGFR marker which indicates that adenovirus has transfected tumor cells (Figure 6). This indicates EPCs carrying replication competent adenoviral vector can transmit viral vector to the surrounding tumor cells and can act as delivery vehicles.

## DISCUSSION

In this study, we used cord blood derived EPCs to

deliver transgenes directly into glioma in rat models. Replication competent adenovirus was used to transfect EPCs and the transfected EPCs were directly injected into the tumors to deliver the transgene to glioma. Adenovirus has been used in gene therapy for the treatment of glioma due to various advantages<sup>[27,40,41]</sup>. Adenoviral vectors have capability to deliver therapeutic genes to tumor cells and at same time replicate in the tumor cells where by it destroys tumor cells<sup>[41]</sup>. However, the efficacy of using adenoviral vectors in tumor therapy is hampered due to volumes of distribution and blood brain barrier<sup>[42,43]</sup>. The migratory ability of the tumor cells and their infiltration into the normal brain parenchyma is the main limiting factor for adenoviral penetration and gene delivery<sup>[44,45]</sup>. New methods are warranted to enhance delivery of therapeutic genes to glioma *via* adenovirus for



**Figure 6 Analysis of transgene delivery to tumor cells by double labeling.** Sections were double with hNIS/vWF or hNIS/EGFR. Sections stain with hNIS/vWF clearly shows some of the red hNIS positive cells also express green vWF positive markers [positive for endothelial progenitor cells (EPC)] indicating the administered EPCs. While hNIS/EGFR double staining clearly shows some of the red positive cells (hNIS positive cells) also express green *EGFR* positive markers indicating expression of transgenes in glioma cells. Prussian blue staining shows the iron positive cells at the corresponding sites.

improved gene delivery. In this context, cord blood derived EPCs have qualities such as their unique ability to self-renew, are easy to extract, give less immune response making them as candidates for gene delivery and recently several genetic tools have been developed to manipulate

them to carry transgenes further, which enhance their case<sup>[14,26,36]</sup>. Our recent studies suggest that cord blood derived EPCs can be used as therapeutic gene delivery vehicles to brain tumors in animal models<sup>[14]</sup>. In our previous report, we showed systemic injection of lentivector



transduced EPCs to carry transgene to glioma. Where we used a replication deficit lentivirus to integrate the reporter gene and showed their deliver capacity of transgenes into tumors<sup>[14]</sup>. In this present study, we have used replication competent adenovirus to transfect EPCs and deliver transgenes directly into tumors *via* intratumor injections. The advantage with this delivery system is one way it can deliver transgenes to tumors and secondly it can destroy the tumor cells by its self-replication properties<sup>[7,23]</sup>. Intratumor administration of cells helps in direct loading of therapeutic genes into tumors and avoids distribution of transfected EPCs to other organs following administration. In addition, adenovirus transfected EPCs can help self-replication of the virus, which helps release a large volume of transgenes into glioma<sup>[41,42,44,46]</sup>. To generate transgenic EPCs, we have transfected EPCs with adenovirus carrying the *hNIS* gene. We tested the transgene expression using Tc-99m uptake assay.

The route of administration of transgenic EPCs is important for the safe and optimal gene delivery. When transduced (by lentivector) EPCs were injected intravenously, most of the transgenic cells accumulate in the liver, spleen, and bone marrow<sup>[14]</sup>. These are vital organs and if it were by replication competent adenoviral vector mediated transfection, adenovirus could have infected the cells in some of these organs<sup>[47,48]</sup>. On the other hand, intratumor injection of EPCs would less likely go beyond the margin of the tumor or the primary organ that contains the tumor. In tumor conditions, tumor cells releases several factors which signals the migration of endothelial cells from bone marrow towards the glioma<sup>[49]</sup>. In this context, intratumor injected EPCs would migrate to the margin of tumors by similar signal mechanisms. In our previous studies we already showed that the locally implanted EPCs migrated towards the periphery of the tumor<sup>[50]</sup>. Several other studies indicate the success of cells delivering viral vectors to tumor, which include mouse fibroblasts, neural precursor cells, T-lymphocytes and MSCs<sup>[51-54]</sup>. To take the cell therapy to clinics it is also important to monitor the migration of the administered cells away from the site of injection using *in vivo* imaging tools. MRI is a non-invasive, high resolution imaging tool for tracking the migration of administered cells *in vivo*<sup>[55]</sup>. Tracking of viral vectors is important aspect in gene therapy to analyze their biodistribution around the glioma and to study their targeting of infiltrative tumor cells<sup>[42]</sup>. Adenoviral vectors targeting glioma has been covalently tagged with super-paramagnetic iron oxide nanoparticles and monitored using MRI<sup>[42]</sup>. We have developed efficient labeling methods to label EPCs using superparamagnetic iron oxide nanoparticles (SPION) and tracked these ferumoxides labeled EPCs *in vivo*<sup>[14,21,36]</sup>. In this study, we infected the EPCs and labeled them with SPIONs and migration and homing of labeled cells was monitored with MRI. We labeled the EPCs instead of labeling the virus, which might have an advantage to generate high signal and easy detection compared to direct labeling. In addition, adenovirus

is free from the conjugation since EPC is labeled with iron particles which help the virus to stay in native form and might increase viral infection ability. High labeling efficiency can be achieved by incubation with SPIONs particles which is rather simple when compared with conjugation<sup>[36]</sup>. In addition, cell labeled with iron oxide as delivery vehicles can be translated to the clinics since SPIONs are FDA approved agents<sup>[56]</sup>. Ahmed *et al*<sup>[57]</sup> used neural stem cells to deliver oncolytic adenovirus to glioma and their results showed NSCs based delivery increased adenovirus survival rate compared with the adenovirus alone. These studies clearly indicate the advantage of using stem cells to deliver the oncolytic adenovirus to tumors<sup>[57]</sup>. To our knowledge, this is the first report to use the cord blood EPCs as adenovirus delivery vehicles to glioma. In addition, we used clinical MRI (3 T) for the monitoring of the distribution of intratumor injected transgenic EPCs which further help in direct translation into clinics. One of the major disadvantage with iron labeling is that it cannot differentiate the dead and live cells, since iron positive signal can be generated from the dead cells, thus it is important to monitor the cell fate in gene therapy approach<sup>[58]</sup>. Moreover, it is important to determine the distribution kinetics of virus at glioma and to determine therapeutic effect as well as subsequent dose calculations<sup>[58]</sup>. To facilitate these qualities, we chose *hNIS* as reporter gene to help in monitoring viable cells, quantitate EPCs engraftment, and to determine the viral load<sup>[58]</sup>.

SPECT imaging was used to track injected cells *in vivo*, and most of the works used In-111 oxine labeling to monitor the cells<sup>[59]</sup>. However, this approach has drawbacks due to radioisotope's (In-111 oxine) half-life and the signal goes down with time, which leads to short term monitoring (up to 7 d) of the injected cells. These short comings can be overcome by using reporter genes such as *hNIS*, which allows repeated detection of injected cells for long periods of time<sup>[7]</sup>. The first report on the use of *hNIS* as a reporter to monitor the delivery of oncolytic adenovirus was the path barker in monitoring and optimizing oncolytic viral therapy<sup>[7]</sup>. Barton *et al*<sup>[7]</sup> showed adenovirus delivery of therapeutic genes (cytosine deaminase/thymidine kinase) along with reporter genes (*NIS*) to monitor the gene therapy. These studies further indicate safety and efficacy of the adenoviral base gene delivery<sup>[5,7,23]</sup>. Most of the work on utilizing *hNIS* to monitor the adenovirus delivery was done on cardiac, prostate, and cystic fibrosis models<sup>[58]</sup> and not much data is available on *NIS* based monitoring of cell based therapies in glioma models. In this study, we transfected EPCs with adenovirus and injected them into the glioma to deliver the transgene (*hNIS*). SPECT imaging detects the uptake of Tc-99m (radioisotope) as long as the cells are alive and express *hNIS*<sup>[7,14,60]</sup>. We used dual imaging modalities to monitor the transgenic EPCs' ability to deliver transgenes and to determine the intratumor homing and migration of administered cells by SPECT and MRI, respectively. For transgene delivery, we observed



radiotracer (Tc-99m) uptake in the tumor site. Histological staining of brain sections revealed presence of iron labeled cells in the tumor not only at the site of injection but also away from the site of injection. We also stained consecutive sections with anti-hNIS antibody and visualized the transgene expression. Double staining of the hNIS stained sections with either vWF or EGFR showed that cells positive for hNIS expression also expressed either vWF or EGFR. These findings indicate that not only endothelial cells, (vWF positive) but also tumor cells (EGFR positive) expressed the transgenes, and this is only possible due the transfected EPCs' ability to deliver the adenoviral vectors in the surrounding tumor cells.

In a conclusion, this study is an exploration of EPCs' capacity to deliver transgenes in glioma upon intratumor administration. We showed transfected EPCs can be tracked once implanted in tumors using MRI imaging. We successfully monitored the transgene delivery by EPCs to tumor cells using SPECT imaging and immunohistochemistry. This study showed the usefulness of EPCs as delivery vehicles of adenoviral vector to deliver therapeutic genes to glioma and act as imaging probe.

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## COMMENTS

### Background

Glioblastoma is one of the aggressive primary brain tumors with survival period falls from one to three years upon treatment. Based on the characteristics of endothelial progenitor cells (EPCs), it is possible to use these cells as vehicles for the delivery of therapeutic genes to gliomas (using viral vectors). In addition, EPCs can be collected from a patient's own peripheral blood and bone marrow, which in turn eliminate the possibility of immune response.

### Innovations and breakthroughs

Authors showed transfected EPCs can be tracked once implanted in tumors using magnetic resonance imaging. Authors successfully monitored the transgene delivery by EPCs to tumor cells using single photon emission computed tomography (SPECT) imaging and immunohistochemistry.

### Applications

The transfected EPCs can be used to deliver the gene in tumor gene therapy. EPCs can be used as both imaging and therapeutic probes.

### Peer review

In this study, authors have shown application of SPECT in monitoring EPCs transfected by recombinant adenoviral vectors harboring hNIS-reporter for the final aim of gene therapy in glioma cells. Results are clear and are informative for publication

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## Pulmonary artery sarcoma successfully treated by right pneumonectomy after definitive diagnosis

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After confirming a definitive diagnosis using a catheter suction biopsy, we successfully performed a right pneumonectomy *via* a median sternotomy without cardiopulmonary bypass. Eighteen months after surgery, no recurrence was observed.

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**Key words:** Pulmonary artery sarcoma; Preoperative diagnosis; Surgery; Catheter suction biopsy; Pneumonectomy

**Core tip:** Pulmonary artery sarcoma (PAS) is a rare and lethal neoplasm that is and usually diagnosed during surgery or autopsy. Early diagnosis and radical surgical resection offer the only chance for survival. However, preoperative histopathological diagnosis is quite difficult owing to the location of the tumor and its rarity. We report a 57-year-old woman patient for whom a preoperative definitive diagnosis of PAS was obtained using catheter-suction biopsy and describe how we successfully performed a curative right pneumonectomy *via* a median sternotomy without cardiopulmonary bypass. Eighteen months after surgery, no recurrence was observed.

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### Abstract

Pulmonary artery sarcoma (PAS) is a rare and lethal neoplasm that is usually diagnosed during surgery or autopsy. Early diagnosis and radical surgical resection offer the only chance for survival. However, making a preoperative histopathological diagnosis is quite difficult. We encountered a 57-year-old woman presenting a PAS that mimicked a pulmonary thromboembolism.

### INTRODUCTION

Pulmonary artery sarcoma (PAS) is a rare tumor that is generally considered fatal and is often misdiagnosed as a





**Figure 1 Axial enhanced computed tomography.** At the level of the bronchus intermedius, showing a filling defect that occupies the entire lumina of the right and interlobar pulmonary arteries.



**Figure 2 Pulmonary angiography.** Showing the flow cutoff of the right main pulmonary artery at the base of the truncus arteriosus.

pulmonary embolism (PE)<sup>[1]</sup>. This misdiagnosis contributes to its poor prognosis, as it delays making the correct diagnosis and administering the appropriate treatment. Only a few hundred cases have been reported<sup>[2]</sup>, following the first description by Mandelstamm in 1923<sup>[3]</sup>. Early diagnosis and radical surgical resection offer the only chance for survival, but owing to the location of the tumor and its rarity, preoperative histopathological PAS diagnosis has seldom been reported<sup>[4,5]</sup>. We report a patient for whom a preoperative definitive diagnosis of PAS was obtained using catheter-suction biopsy and describe how we successfully performed a curative right pneumonectomy.

## CASE REPORT

A 57-year-old female was referred to our hospital with an abnormal shadow on chest radiography and a history of chest pain, dyspnea, malaise, and fever. Her medical history included osteoporosis and scoliosis. Laboratory test results revealed a mild impairment of liver function, elevation of biliary enzymes and moderate increase of the erythrocyte sedimentation rate. A remarkable prolongation of the activated partial thromboplastin time (180/30.5 s) and a slight acceleration of the fibrino-

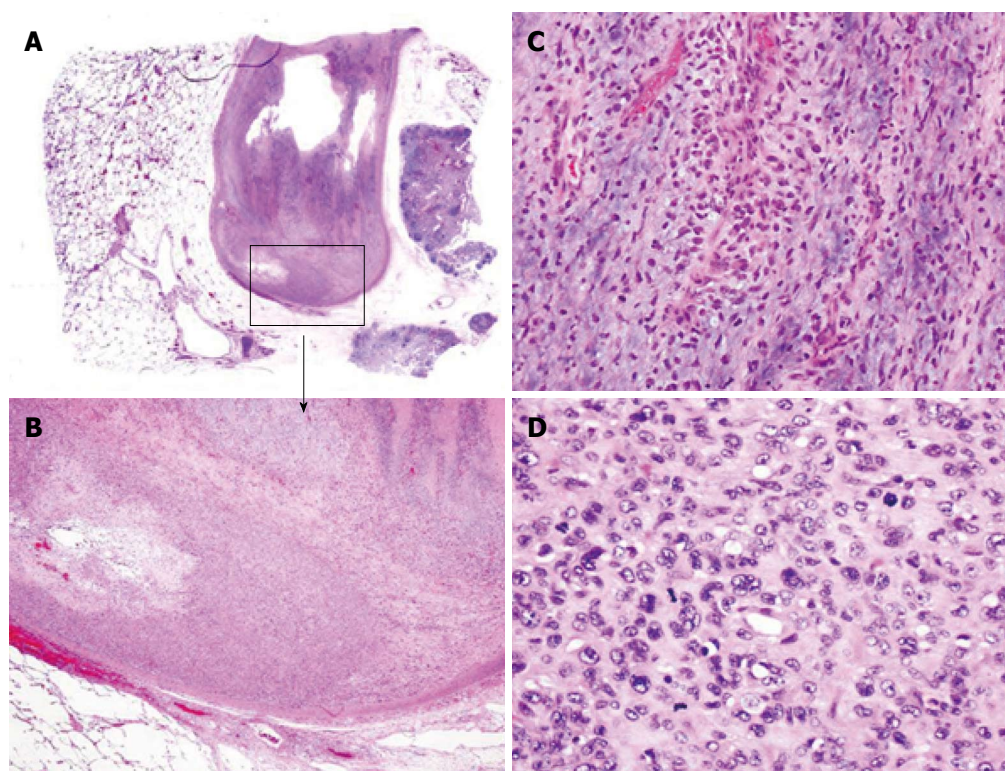


**Figure 3 Lung perfusion scintigraphy.** Just prior to the operation (right side) had worsened relative to that of a previous examination (left side).

gen (618 mg/dL) and fibrin degradation products (6.4  $\mu$ g/mL), as well as D-dimer (2.50  $\mu$ g/mL), were also detected. She was initially diagnosed with a pulmonary embolism according to the enhanced computed tomography (CT) findings and underwent thrombolytic therapy with the placement of an inferior vena cava (IVC) filter. However, her symptoms and CT findings did not improve with treatment (Figure 1), and the IVC filter was retrieved. Moreover, positron emission tomography-computed tomography (PET/CT) revealed moderate <sup>18</sup>F-fluorodeoxyglucose uptake in the pulmonary embolism. We then considered the possibility of PAS and performed a pulmonary angiography, which revealed a filling defect occupying the entire luminal diameter of the right main pulmonary artery at the base of the truncus anterior (Figure 2). We performed intravenous catheter-suction biopsy using a 9 F multipurpose-guiding catheter (Vista Brite Tip®, Cordis Corporation, East Bridgewater, NJ) and a 50 mL syringe at the right peripheral pulmonary artery. The proximal portion of the lesion, which mostly consisted of a blood clot, was too soft to perform a traditional biopsy. Pathology revealed a few atypical spindle cells in a large volume of clotted blood; according to these results, the lesion was definitively diagnosed as PAS. While awaiting surgery, the patient had a recurrence of chest pain and fever; we suspected that these symptoms were due to a repeat pulmonary infarction and that her initial symptoms indicated a prior infarction. We therefore performed lung perfusion scintigraphy. Compared with a previous examination performed 1.5 mo prior, the findings were obviously worsened: the right lung was not visualized on the scan (Figure 3).

We performed a right pneumonectomy through a median sternotomy, which is suitable for exposing the right main pulmonary artery between the ascending aorta and the superior vena cava without using an artificial cardiopulmonary machine. We transected the pulmonary artery after double-stapling at its origin, and the right main bronchus was also stapled at this location. The pulmonary veins in the right thoracic cavity were stapled. We did not carry out mediastinal lymph node dissection.





**Figure 4 Hematoxylin and eosin microscopy.** A, B: The tumor occupies the lumen of the pulmonary artery (A: loupe image, B:  $\times 20$  magnification); C: Tumor cells consisting of atypical spindle cells ( $\times 200$  magnification); D: Tumor cells accompanying scattered mitotic figures ( $\times 400$  magnification).

Macroscopically, a yellowish-white tumor was observed within the lumen of the right pulmonary artery, and diffuse lung congestion was noted. Microscopic examination revealed many atypical polymorphic spindle cells in the lumen of the pulmonary artery; the pathological diagnosis was intimal sarcoma of the pulmonary artery (Figure 4). The postoperative course of the patient was uneventful, and she was discharged 10 d after surgery. Eighteen months later, no evidence of recurrence was observed on CT angiography (Figure 5).

## DISCUSSION

PAS is an extremely rare and usually lethal neoplasm that is most commonly diagnosed during surgery or autopsy. It is often misdiagnosed as PE<sup>[1]</sup>, and its prognosis is very poor, partially due to this misdiagnosis precluding more rapid treatment. Because the tumor always arises from the central pulmonary arteries, preoperative histopathological diagnosis is quite difficult and has only rarely been reported<sup>[4,5]</sup>. We used transvenous catheter-suction biopsy that required repeated suction attempts using a syringe during pulmonary angiography because few tumor cells were present within an area of extensive coagulation. With a definitive diagnosis, we successfully performed a curative right pneumonectomy, and the patient has been in good health without recurrence for 1 year and 6 mo after surgery.

There are a few reasons why we chose right pneumonectomy as the curative operation for this patient.



**Figure 5 Axial enhanced computed tomography.** Eighteen months after surgery, no recurrence is apparent.

The first was the deterioration of her right lung, with a diffuse right lung infarction that had been ongoing for almost 1 year. The patient had gradually become accustomed to this cardiopulmonary status, with a narrowing of the right thorax already having occurred by the time she was referred to our hospital. We therefore decided that right pneumonectomy would be reasonable for this patient. We also thought that right pneumonectomy was necessary to achieve complete tumor resection, as tumor cells may have existed in the distal pulmonary artery-lumen coagulation. Gan *et al*<sup>[6]</sup> reported that patients with PAS who undergo distal embolectomy live longer than patients who do not, suggesting that PAS tumor cells exist in the distal pulmonary artery thrombi.

Differentiating between PAS and PE is extremely difficult. Our strongest reason for suspecting PAS was the enlargement of the pulmonary artery diameter on CT, which increased despite anticoagulant therapy. Yi *et al*<sup>[7]</sup> evaluated 7 patients with PAS and reported that CT can help differentiate PAS from PE by indicating a low-attenuation filling defect that occupies the entire luminal diameter of the proximal or main pulmonary artery in PAS. This finding was observed in all 7 patients (100%), and expansion of any segment of the pulmonary artery, with an extensive intraluminal filling defect, was observed in 6 of the 7 patients (86%). Moreover, Cox *et al*<sup>[2]</sup> described that the presence of a hilar mass causing unilateral enlargement of the pulmonary artery and proximal branches is specific to pulmonary artery sarcoma. Unilateral central embolus is uncommon. In patients diagnosed with PE, the possibility of PAS should be considered if a unilateral widened diameter of the pulmonary artery and/or a low-attenuation filling defect occupying the entire luminal diameter at the level of the main or proximal pulmonary artery is present.

In our patient, the 2 bouts of back pain and high fever were thought to be due to a major pulmonary infarction. The visible progression of her disease on lung perfusion scintigraphy over a 1.5-mo period was valuable in the decision of the appropriate pulmonary-artery transection site through a median sternotomy. If a patient presents suspicious symptoms that might indicate advancing PAS, clinicians should not hesitate to carry out additional examinations.

In a conclusion, PAS is a rare, life-threatening tumor that arises from the pulmonary artery and its proximal branches. The most effective treatment (and the best chance for sur-

vival) is radical surgery. We treated a patient with PAS using a right pneumonectomy after making a definitive diagnosis using catheter suction biopsy. The patient is alive and well, 18 mo after surgery.

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfeide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee.

Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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