Experimental and Molecular Pathology 102 (2017) 347–353

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Gli1 expression in cancer stem-like cells predicts poor prognosis in patients with lung squamous cell carcinoma

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ARTICLE INFO

Article history:
Received 16 January 2017
and in revised form 3 March 2017
Accepted 8 March 2017
Available online 09 March 2017

Keywords:
Gli1
Cancer stem cell
Lung squamous cell carcinoma
Prognosis

ABSTRACT

Purpose: Glioma-associated oncogene homolog 1 (Gli1) is involved in cancer stem cell (CSC) maintenance in various tumors; however, its expression and clinical significance in lung squamous cell carcinoma (LSCC) has not been reported. In this study, we aimed to reveal the clinical significance of Gli1 in LSCC and investigate the potential of Gli1 as a CSC marker by comparing its expression with that of other stemness-related genes in LSCC.
Methods: We assessed the expressions of Gli1, LSD1, CD44, Sox9 and Sox2 by immunohistochemistry in the tissue specimens obtained from 101 patients with LSCC. The relationship of Gli1 expression with clinicopathological parameters and cell-cycle regulating genes was investigated.
Results: Gli1 expression was significantly correlated with T stage ($P < 0.001$), lymph node metastasis ($P = 0.002$), and clinical stage ($P = 0.005$) of LSCC. The Kaplan-Meier survival analysis revealed that the expression of Gli1 in LSCC was all significantly associated with poor overall survival (OS: $P = 0.005$). Cox regression analysis further confirmed that Gli1 is a prognostic marker of unfavorable clinical outcome of LSCC. Gli1 expression was significantly correlated with the expression of stemness-related genes such as LSD1 ($P = 0.009$) and CD44 ($P < 0.001$), but not with those of Sox2 and Sox9. However, Gli1 expression was associated with the expression of hypoxia-inducible factors1α (HIF1α; $P < 0.001$) and Cyclin D1 ($P = 0.002$), respectively. In additionally, microvessel density (MVD) was significantly higher in Gli1-positive LSCC than in the negative LSCC ($P = 0.026$).
Conclusions: Our results suggest that Gli1 may be a potential LSCC stem cell marker and an independent indicator of poor prognosis for patients with LSCC.

1. Introduction

The most common causes of cancer-related deaths in men and women is lung cancer accounting for more than one-quarter (27%) of global cancer-related deaths. Non-small cell lung cancer (NSCLC) accounts for >85% of all lung cancer cases. There have been limited improvements in NSCLC therapy over the past few decades, and a 5-year survival rate of only 18% has been estimated for patients with NSCLC (Siegel et al., 2016). Small cell lung cancer (SCLC), accounting for approximately 15% of all lung cancers, is one of the most aggressive subtypes and has a 5-year survival rate of <10% (Siegel et al., 2016).

Cancer stem cells (CSCs) or cancer-initiating cells are small subpopulations of cancer cells, which can differentiate and generate heterogeneous cell populations to constitute the tumor (Visvader and Lindeman, 2008). CSCs thus play a key role in the initiation and progression of malignant tumors (Visvader and Lindeman, 2008). Conventional therapies that kill the bulk of tumor may ultimately fail to specifically target CSCs. Thus, specifically targeting and eliminating CSCs may be an alternative for developing new therapeutic approaches to treat cancer (Visvader and Lindeman, 2008). NSCLC tumors contain stem cells like responsible for the initiation, maintenance, relapse, and metastasis of lung tumors. (Justilien et al., 2012). These cells exhibit resistance to commonly used therapeutic agents (Chen et al., 2008), making them a likely cause of therapeutic failure.

Hedgehog (HH) signaling pathway specifies the proliferation, differentiation and migration of normal stem cells (Varjosalo and Taipale, 2008). Aberrantly activated HH signaling pathway also plays an important role in the initiation and development of lung cancer and is required for the maintenance of lung CSCs (Rodriguez-Blanco et al., 2013; Hong et al., 2014; Bora-Singhal et al., 2015). This pathway is composed of HH ligands, HH receptors (Ptch), Smoothened (Smo), and Gli proteins (Robbins et al., 2012). Gli1 protein is a vital transcription factor of HH signaling pathway and contributes to the activation of downstream genes of HH pathway. Aberrant activation of HH signaling is associated with multifarious human tumors where the pathway is implicated in tumorigenesis, malignancy, metastasis and CSCs (Ruiz,

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http://dx.doi.org/10.1016/j.yexmp.2017.03.004
0014-4800/

2008). In recent years, there has been an increasing amount of evidence to support a CSC phenotype in human lung cancer (Eramo et al., 2008). However, little is known regarding the prevalence and clinical implications of Gli1 in patients with lung squamous cell carcinoma (LSCC).

In the present study, to identify Gli1 as a potential LSCC stem cell marker, Gli1 expression was examined by immunohistochemistry on tissue microarray slides from 81 human LSCC and 20 adjacent non-tumor lung tissues. To confirm the stem cell-like characteristics of Gli1-positive cells, we analyzed and compared its expression with that of other stemness-related genes such as CD44, LSD1, Sox2, and Sox9. We showed here that Gli1 expression not only indicates poor prognosis for LSCC and is a potential marker for LSCC stem cells.

2. Materials and methods

2.1. Tissue specimens

A total of 101 formalin-fixed and paraffin-embedded tumor tissue samples including 81 LSCC and 20 adjacent non-tumor lung tissue were obtained from patients who underwent lung surgery and were stored in Shanghai Outdo Biotech Co. Ltd. (Outdo Biotech). No patient received preoperative chemotherapy or radiotherapy. Clinical and pathological reports were reviewed for age, sex, tumor size, histological grade, invasion depth (pT), nodal status (pN), and distant metastasis (pM). The pTNM classification was applied according to guidelines from the 2010 American Joint Committee on Cancer staging manual (AJCC 7th edition).

2.2. Immunohistochemical staining procedure

Sections on microslides were deparaffinized with xylene, hydrated using a diluted alcohol series, and immersed in 3% H_2O_2 in methanol to quench endogenous peroxidase activity. Sections were treated with TE buffer (10 mM Tris and 1 mM EDTA, pH 9.3) at 98 °C for 30 min. To reduce non-specific staining, each section was blocked with 4% bovine serum albumin in PBS with 0.1% Tween 20 for 30 min. The sections were then incubated with anti-Gli1 monoclonal antibody (1:100, Abcam, UK), anti-LSD1 (1:250, Sigma, USA), anti-Sox2 (1:100, R&D, USA), anti-Sox9 monoclonal antibody (1:100, Abnova, USA), anti-CD44 (1:100, ZSGB-BIO, China), anti-P21 (1:100, Big, USA), anti-CyclinD1 (1:100, Big, USA), anti-pAkt-Thr308 (1:100, Abcam, UK), anti-pAkt-Ser473 (1:100, Abcam, UK), NF-κB (1:100, CST, USA) and LC3A(1:500, CST, USA) in PBST containing 3 mg/ml goat globulin (Sigma, St. Louis, MO, USA) for 60 min at room temperature, followed by three successive washes with buffer. The chromogen used was ImmPACT AEC Peroxidase Substrate (VECTOR Laboratories) for 20 min. Sections were counterstained with Meyer's hematoxylin. After reading and taking photograph the slide, sections were then used stripping buffer (20% SDS, 0.5 M Tris, and mercaptoethanol) to removing the original antibody for 1 h at the water bath of 56 °C and then for 10 min dehydrated alcohol to removing the red reaction, so that the sections can be used again. Omitting the primary antibody provided negative controls for immunostaining.

The double immunostaining procedure was performed using a two-step method with Gli1, LSD1, CD44 antibodies (developed with 3, 3′-diaminobenzidine) (brown reaction product) and anti-CD105 antibodies (1:250, Abcam, Cambridge, UK) (developed with ImmPACT AEC Peroxidase Substrate) (red reaction product) to observe the relationship between the expression of Gli1/CD105 and microvessel density (MVD) in ESCC. Primarily, for the Gli1, LSD1 and CD44 protocols, except that the chromogen with the 3, 3′-diaminobenzidine (Dako) for 10 min (FLEX20), all steps are the same. Then, subsequent staining of the same section was performed after incubating the samples with an antibody to CD105 by ImmPACT AEC Peroxidase Substrate for 20 min.

2.3. Statistical analysis

Correlations were examined using Pearson's chi-square test as appropriate, correct for multiple testing for the calculation of the correlations coefficients using Bonferroni method, overall survival (OS) were determined using the Kaplan–Meier method and were compared using the log-rank test. Survival was measured from the date of surgery.

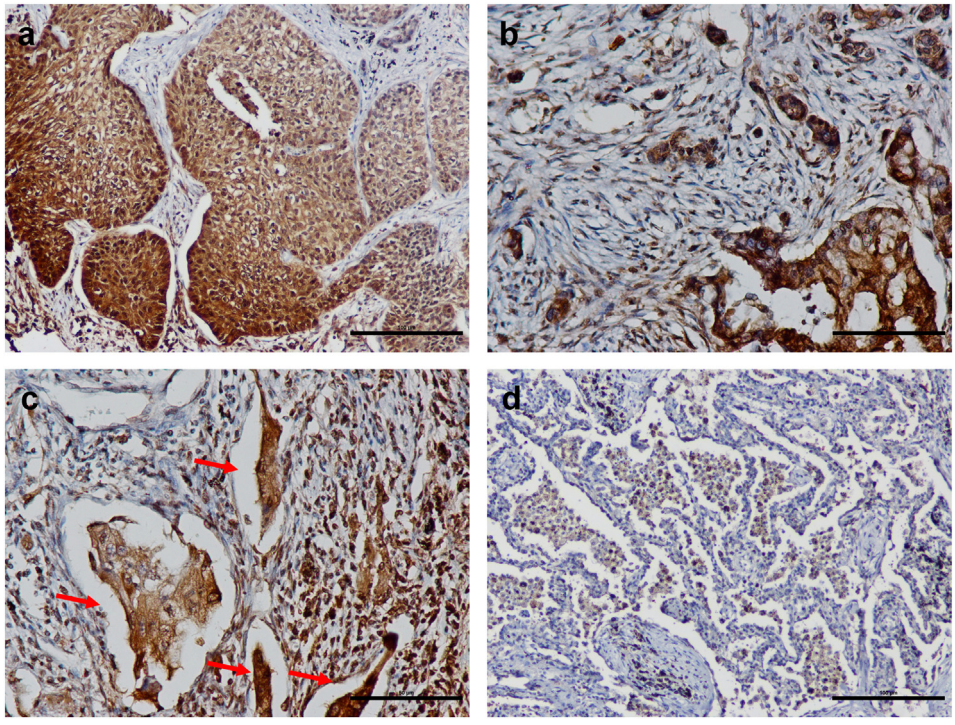

Fig. 1. Expression and distribution of Gli1 in LSCC tissues (well differentiated (a) and poorly differentiated (b) and lymphatic invasion area (c)) and normal lung tissues (d), detected by immunohistochemistry (×100).