Pathology – Research and Practice 211 (2015) 939–947

Contents lists available at ScienceDirect

Pathology – Research and Practice

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

Original article

Clinicopathologic features and prognostic implications of Gankyrin protein expression in non-small cell lung cancer

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ARTICLE INFO

Article history:
Received 5 March 2015
Received in revised form 14 August 2015
Accepted 9 September 2015

Keywords:
Gankyrin
Oncoprotein
Lung cancer
NSCLC
Survival
Prognosis
Immunohistochemistry

ABSTRACT

Purpose: The expression of Gankyrin, a liver cancer-related oncoprotein, has been observed in several human malignancies including non-small cell lung cancer (NSCLC). However, the clinic relevance of Gankyrin expression in NSCLC remains unclear.
Methods: Gankyrin expression was assessed using immunohistochemical (IHC) methods in 166 paired paraffin-embedded NSCLC specimens and adjacent normal tissues. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and Western blotting were employed to measure the expression of Gankyrin in 24 paired fresh NSCLC specimens and the corresponding normal tissues. The association of Gankyrin expression with clinicopathological parameters was also evaluated. Kaplan–Meier survival analysis and Cox proportional hazards models were used to estimate the effect of Gankyrin expression on survival.
Results: Data showed that Gankyrin was expressed in 78.3% (130/166) and 28.9% (48/166) of cancer lesions and corresponding adjacent normal tissue, respectively. And the Gankyrin overexpression in tumor tissue occurred in 53.6% (89/166) of patients, while overexpression of Gankyrin in normal tissue occurred only in 4.8% (8/166) of patients ($P < 0.001$). Semi-quantitative RT-PCR and Western blotting showed that NSCLC specimens had increased Gankyrin mRNA and protein expression compared to the corresponding normal tissues. Out of all the clinicopathological factors analyzed, Gankyrin overexpression was significantly correlated with lymphatic metastasis ($P < 0.001$) and p-TNM stage ($P < 0.001$). Gankyrin-overexpressed NSCLC patients had a significantly shorter survival time ($P < 0.001$, Log-rank test), and the prognostic significance of Gankyrin overexpression was apparent in both squamous cell carcinoma patients ($P = 0.028$) and adenocarcinoma patients ($P < 0.001$). Multivariate analysis indicated that Gankyrin overexpression may be an independent prognostic factor in NSCLC (hazard ratio [HR], 1.51; $P = 0.041$).
Conclusion: Our results indicate that Gankyrin overexpression is of clinical significance and can serve as a prognostic biomarker in NSCLC.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) accounts for most cases of lung cancer. However, the long-term survival rate of NSCLC patients remains unsatisfactory. A majority of NSCLC patients die from recurrent disease and distant metastases even after undergoing curative surgical resection [1–3]. There is an urgent need to identify new prognostic markers that can facilitate a better assessment of the survival probabilities and optimized therapies for individual patients.

Gankyrin, also known as p28GANK, is a small ankyrin-repeat protein discovered first in hepatocellular carcinoma [4], and can induce anchorage-independent growth and tumorigenicity in NIH/3T3 cells [4,5]. The previous research has confirmed that Gankyrin is overexpressed in hepatocellular carcinoma (HCC), and participates in the tumorigenicity and metastasis of this malignancy [4,6–9]. In addition, overexpression of Gankyrin was also

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http://dx.doi.org/10.1016/j.prp.2015.09.010
0344-0338/

detected in other malignant diseases, including esophageal cancer [10], colorectal cancer [11], pancreatic cancer [12], breast cancer [13], human cholangiocarcinoma [14], endometrial carcinoma [15], cervical cancer [16], oral cancer [17] and lung cancer [5], which indicates that Gankyrin might be a conservative protein playing essential roles in tumor growth and progression. For lung cancer, increased Gankyrin expression was required for the constitutive activation of Akt and tumorigenesis in those who have ras mutation [5]. However, the prognostic role of Gankyrin in NSCLC was not found.

To study the clinicopathologic features and prognostic implications of Gankyrin expression in patients with NSCLC, we investigated the expression of Gankyrin in NSCLC by IHC staining, Semi-quantitative Reverse transcription polymerase chain reaction (RT-PCR) and Western blot, and assessed the relationships between Gankyrin expression and clinical parameters.

Materials and methods

Patients and tissue samples

Paraffin-embedded tissue specimens from 166 patients with confirmed NSCLC, collected from 2006 to 2009, were analyzed from an archived thoracic oncology tissue repository at the Department of Thoracic Surgery of Tangdu Hospital. 24 fresh NSCLC tissue specimens and the paired adjacent normal lung tissues were also obtained from patients undergoing radical surgery at the same center. Detailed information was obtained from the medical records of the enrolled patients in a computerized registry database, including patient age, gender, clinical manifestation, surgical method, tumor status, histological differentiation, nodal status and follow-up information. Follow-up lasted through 01 July, 2014, with a median follow-up period of 62 months for living patients (range, 60–81 months). The day of surgery was considered as the starting day for estimating postoperative survival time. Histological classification of tumors was reviewed by pathologists and based on the World Health Organization criteria. All tumors were staged according to the pathological tumor/node/metastasis (p-TNM) classification (7th edition) of the International Union against Cancer [18]. The study protocol was approved by the Regional Ethics Committee for Clinical Research of the Fourth Military Medical University. All patients provided written informed consent for use of their medical records and tissue specimens for research purposes.

Immunohistochemistry

We studied the whole tissue slides. The tissue blocks were cut into 5-μm sections and mounted on silane-coated slides. The slides were then dewaxed in xylene and rehydrated through a graded series of ethanol solution. Endogenous peroxidase activity was blocked by immersing the slides in a solution of 3% hydrogen peroxide in methanol for 30 min. Antigen retrieval was performed by microwaving sections in 10 mM citrate buffer (pH 6.0) at 95 °C for 20 min. To reduce nonspecific binding, slides were blocked with goat serum for 30 min. Then, the sections were incubated in a humidified chamber at 4 °C overnight with primary anti-gankyrin (diluted 1:50, Santa Cruz Biotechnology, Inc. USA) antibodies. After washing three times in PBS (phosphate-buffered saline), the slides were incubated for 60 min with a labeled polymer En Vision+. Peroxidase activity was visualized with the DAB Elite kit (Dako, Denmark), and the slides were counterstained with hematoxylin. To confirm the specificity of the immunostaining, negative controls were obtained by replacing the primary antibody with PBS.

Evaluation of immunohistochemical staining

Five random fields from each section were viewed under a light microscope (Leica DM4000B, Germany) at ×200 magnification. The expression of Gankyrin was scored by multiplication of the percentage of positive tumor cells and the staining intensity. Initially, the percentage of positive cells was scored as 0 (0%), 1 (1–10%), 2 (11–50%), and 3 (51–100%). Thereafter, intensity of staining was scored as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). By ROC analyses, the case with a total scores ≥4 was classified as overexpression (Sensitivity 84.6, Specificity 77.2), and the cases with a total scores <4 were classified as weak (the final score = 3) or negative expression (the final score < 3). Both overexpression and weak expression were considered as positive expression. All slides were assessed by three independent investigators who were blinded to the clinical features and outcomes. The final immunohistochemical staining score reported is the average of the scores from the three investigators. No significant conflicting results were found in the scoring process.

Purification of total cellular mRNA and semi-quantitative RT-PCR

Total RNA was extracted from the fresh tissue specimen of NSCLC patients using E.Z.N.A.®Total RNA Kit I (Omega Bio-Tek Inc., Georgia, USA) according to the manufacturer's protocols. Reverse transcription of total cellular RNA was performed using a RevertAid First-Strand cDNA Synthesis Kit (Thermoscientific, Vilnius, Lithuania). cDNA was subjected to PCR for 35 cycles of amplification using an cDNA PCR kit (CWbiotech, Peking, China). Each PCR cycle consisted of a denaturation step for 45 s at 95 °C, an annealing step for 35 s at 62 °C, and an extension step for 50 s at 72 °C. The PCR products were separated on 1.0% agarose gel and visualized by ethidium bromide staining. Beta-actin mRNA was used as an internal control for semi-quantitative analyses of gankyrin mRNA. The PCR primers used for Gankyrin were 50-AGCAGCCAAGGGTAACTTGA-30 as the forward primer and 50-TACTTGCTCCTTGGGACACC-30 as the reverse primer; and for beta-actin, 50-CTCCATCCTGGCCTCGCTGT-30 was used as the forward primer and 50-GCTGTCACCTTCACCGTTCC-30 as the corresponding reverse primer.

Western blot

Fresh tissues were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed on ice in RIPA (radio immunoprecipitation assay) buffer (Cell Signaling Technology, Danvers, MA, USA) containing complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). After being centrifuged at 14,000 for 20 min, total protein was denatured at 65 °C for 30 min. Protein from tissues was separated via SDS–PAGE and transferred to a PVDF membrane (GE healthcare, USA). Membranes were blocked with 9% fat-free milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 3 h at room temperature; the membranes were then incubated overnight at 4 °C with anti-Gankyrin (diluted 1:200, Santa Cruz Biotechnology, Inc. USA), or anti-beta actin (1: 2500, Abcam, Cambridge, MA, USA) antibodies. Followed by anti-rabbit horseradish peroxidase conjugated IgG (BIOS 1:5000), an ECL kit (GE healthcare, USA) was used for detection.

Statistical analysis

Associations between Gankyrin overexpression and clinicopathological parameters were evaluated using the χ^2 test. Survival was examined using the Kaplan–Meier method, and the significance of the difference was evaluated using the log-rank test. Correlation analyses of the survival time and various