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Short Communication

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The chimeric transcript RUNX1–GLRX5: a biomarker for good postoperative prognosis in Stage IA non-small-cell lung cancer

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Abstract

Stage IA non-small-cell lung cancer cases have been recognized as having a low risk of relapse; however, occasionally, relapse may occur. To predict clinical outcome in Stage IA non-small-cell lung cancer patients, we searched for chimeric transcripts that can be used as biomarkers and identified a novel chimeric transcript, RUNX1–GLRX5, comprising RUNX1, a transcription factor, and GLRX5. This chimera was detected in approximately half of the investigated Stage IA non-small-cell lung cancer patients (44/104 cases, 42.3%). Although there was no significant difference in the overall survival rate between RUNX1–GLRX5-positive and -negative cases ($P = 0.088$), a significantly lower relapse rate was observed in the RUNX1–GLRX5-positive cases ($P = 0.039$), indicating that this chimera can be used as a biomarker for good prognosis in Stage IA patients. Detection of the RUNX1–GLRX5 chimeric transcript may therefore be useful for the determination of a postoperative treatment plan for Stage IA non-small-cell lung cancer patients.

Key words: non-small-cell lung cancer, gene fusion, biological markers, RUNX1, GLRX5

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (1). More than 80% of lung cancer cases are of non-small-cell lung cancer (NSCLC), and for early-stage NSCLC, surgical resection provides the best prognosis. Postoperative adjuvant chemotherapy is effective for Stage IB or higher NSCLC, but its effect on Stage IA NSCLC has not been established (2–4). The postoperative 5-year survival rate for Stage IA NSCLC patients is ~80% (5,6); 20–30% of cases eventually relapse despite radical surgical resection (7). Predicting the prognosis of Stage IA patients immediately after surgery can help determine an appropriate postoperative treatment plan such as adjuvant chemotherapy.

Recently, many chimeric genes or transcripts have been identified in solid tumors (8–11), and some of these are correlated to prognosis, such as the PJA2-FER chimeric transcript, which we previously found to be correlated with poor postoperative survival in NSCLC (12,13). In this study, we identified a novel chimeric transcript, RUNX1–GLRX5, in Stage IA NSCLC patients. We found that detection of RUNX1–GLRX5 predicts a good postoperative prognosis and can thus be a useful biomarker.

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Patients and methods

Patients and samples

The samples were collected from Stage IA NSCLC patients who underwent surgical resection at the University of Tokyo Hospital, Japan. Twenty-nine Stage IA patients between June 2005 and December 2006 were recruited. Validation was then performed in 104 Stage IA patients between June 2005 and November 2009. The study was approved by the ethics committee of the hospital, and written informed consent was received from all patients. The exact date and clinicopathological characteristics of all patients were gathered from medical records. Relapse-free survival was measured from the period of surgery until disease relapse or metastasis, and overall survival was measured from the period of surgery until the date of death.

Rapid amplification of cDNA ends

Rapid amplification of cDNA ends (RACE) products were prepared from clinical samples using the GeneRacer Kit (Life Technologies, Carlsbad, CA) and KOD plus V.2 (Toyobo, Osaka, Japan). Based on the longest isoform variant 1 sequence of RUNX1 (NM_001754.4), a GSP and a nested GSP for 3′RACE RUNX1 were designed as follows: GSP, 5′-AGAAGTCTGAACCCAGCATAGTGGTC-3′, and nested GSP, 5′-AGGGTTTCGCAGCGTGGTAAAAG-3′. The RACE products were sequenced using a previously reported method (13).

RT-PCR to detect chimeric RUNX1–GLRX5

The detection of RUNX1–GLRX5 in each clinical sample was determined using RT-PCR using cDNA as a template. The total RNA from each clinical sample was isolated using RNAiso (TaKaRa, Shiga, Japan), and human lung total RNA was purchased from Life Technologies. A reverse transcription reaction was run using SuperScript III reverse transcriptase (Life Technologies). RT-PCR was performed using a forward primer designed to target exon 2 of RUNX1 (5′-TTCCTT CGTACCCACAGTGCTTC-3′) and a reverse primer designed to target exon 2 of GLRX5 (5′-AAGTCCCCATTCTGGTGCATCTG-3′). Amplification was performed using AmpliTaq Gold 360 Master Mix (Life Technologies).

Search for chimeric variant patterns of RUNX1–GLRX5

RT-PCR was performed to search for the chimeric RUNX1–GLRX5 variant. The sequence of each primer of RUNX1 was as follows: exon 3, 5′-GGAATGAATCCTTCTAGAGACGTCCAC-3′; exon 4a, 5′-GGCTTGTTGTGATGCGTATCCC-3′; exon 4b, 5′-AAGACCCT GCCCATCGCTTTC-3′; exon 5, 5′-CTCGGCTGAGCTGAGAAA TGCTAC-3′; exon 6, 5′-CAAGTCGCCACCTACCACAG -3′; exon 7, 5′-ACCACTCCACTGCCTTTAACCC-3′; exon 8, 5′-ATATTTA GCATGGATCCCGGTACAC-3′; exon 9, 5′-CCGTGGTCCTACGA TCAGTCCTAC-3′; and exon 10, 5′-CACCCGACCTGACAGC GTTC-3′.

Expression level analysis of RUNX1 and GLRX5

The expression of RUNX1 and GLRX5 mRNA in each clinical sample was analyzed by quantitative RT-PCR using Thunderbird qPCR Mix (Toyobo). The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde-3-phosphate dehydrogenase as the internal control. The following primers were used: RUNX1, 5′-GGGATGTTCCAGATGGCACTC-3′/5′-TTTTGATGGCTCTGT GGTAGGTG-3′, and GLRX5, 5′-AAGGACAAGGTGGTGGTCT TC-3′/5′-TCAGTTCTTCCACCAAGTCCC-3′.

Plasmid vector constructions

The RUNX1–GLRX5 sequence was extracted from the 3′RACE products of RUNX1. After ligation into a pGEM-T Easy Vector System (Promega, Madison, WI), the insert was removed with the restriction enzyme NotI (New England Biolabs, Ipswich, MA). The lentivirus vector CSII-CMV-MCS-IRES2-Bsd was received from the RIKEN BioResource Center, Japan. A blank vector that did not contain the insert was created as a control.

Evaluation of the growth rate of RUNX1–GLRX5-expressing cells

A RUNX1–GLRX5-negative lung cancer cell line (A549) was purchased from the American Type Culture Collection (Manassas, VA). We force-expressed RUNX1–GLRX5 in these cells using the lentivirus vector and evaluated the growth rate of the cells as follows: 5×10^4 cells were seeded onto 10 cm plates (day 0). The number of cells was counted three times for each sample on Days 2, 4, 6, 8 and 10. The natural logarithm of the number of cells was plotted on the vertical axis, whereas the elapsed time was plotted on the horizontal axis. A linear regression analysis was performed for the period of exponential growth. The slope was then considered to be the growth rate.

Statistical analysis

The χ^2 test and the unpaired *t*-test were used to analyze differences in the clinicopathological characteristics, age, and mRNA expression levels of RUNX1 and GLRX5 (ΔΔCt value) between the RUNX1–GLRX5-positive cases and the RUNX1–GLRX5-negative cases. Relapse-free and overall survival curves were calculated using the Kaplan–Meier method and were compared using a log-rank test. To assess the contribution of the expression level of RUNX1 and GLRX5 on the relapse-free period, a univariate Cox proportional hazards analysis was performed with the expression levels as explanatory variables. SPSS II (SPSS, Chicago, IL) software was used for analysis and the significance level was set at $P < 0.05$.

Results

Search process

To identify novel chimeric transcripts containing a transcription factor or a tyrosine kinase, which have important biological functions in cancer, we performed the RACE in NSCLC samples and identified the RUNX1–GLRX5 chimeric transcript in which exon 2 of RUNX1, a transcription factor (14), was fused to the 5′-end of exon 2 of GLRX5 (Fig. 1A). This chimeric transcript has not been previously reported. The RUNX1–GLRX5 sequence was in-frame from the start codon of exon 2 of RUNX1, through the junction, to the stop codon of exon 2 of GLRX5, and thus, it was assumed to be translated into a chimeric protein of 85 amino acids. To search for the presence of other possible chimeric patterns, RT-PCR was performed with forward primers designed for each exon of RUNX1, but no other variant was identified. RUNX1–GLRX5 was present in 14 of 29 Stage IA cases (48.3%; Fig. 1B) and was not detected in the RNA of normal human lungs. All four fatal Stage IA cases were RUNX1–GLRX5 negative. The overall survival analysis also showed that RUNX1–GLRX5-positive cases had a better prognosis than RUNX1–GLRX5-negative cases (log-rank test: $P = 0.049$; Fig. 1C).

We cloned RUNX1–GLRX5 into a lentiviral vector and infected it into A549 cells, but there was no significant change in the growth rate of the cells (specific growth rate was 0.83 day^{-1} in controls and 0.88 day^{-1} in the RUNX1–GLRX5-expressing cells; $P = 0.8$).

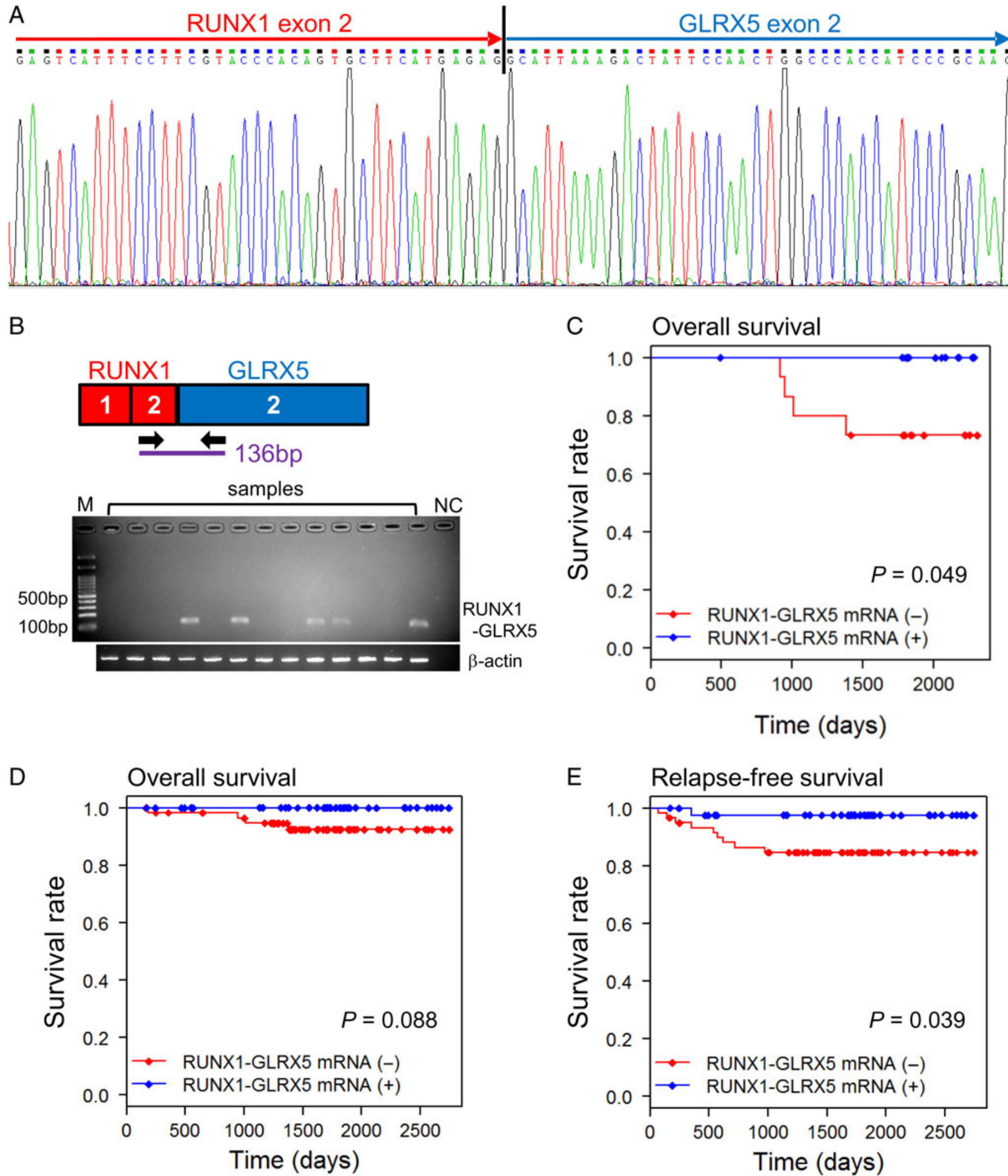

Figure 1. Identification and validation of a novel RUNX1–GLRX5 chimeric transcript. (A) The sequencing results of the PCR band of a RUNX1–GLRX5-positive sample, showing exon 2 of RUNX1 fused to exon 2 of GLRX5. (B) Screening for RUNX1–GLRX5 mRNA in each sample and in a negative control (NC) (water). The black arrows in the upper diagram represent the primers used to detect RUNX1–GLRX5. The numbers show the exon number; exons of RUNX1 and GLRX5 are shown in red and blue, respectively. A representative photograph of electrophoresis of RT-PCR products is shown. Marker (M), 100 bp DNA ladder. (C) The overall survival curve of 29 Stage IA NSCLC patients. The group with RUNX1–GLRX5-positive cases has a significantly better prognosis (log-rank test: $P = 0.049$). (D) The overall survival curve of 104 stage IA NSCLC patients. (E) The relapse-free survival curve of the same group. Although a significant difference in the overall survival rate in relation to the detection of RUNX1–GLRX5 was not observed (log-rank test: $P = 0.088$), the relapse rate of RUNX1–GLRX5-positive cases was significantly lower compared with that of RUNX1–GLRX5-negative cases (log-rank test: $P = 0.039$).

Validation process

Next, we verified the hypothesis that RUNX1–GLRX5 may be a prognostic biomarker for Stage IA NSCLC. Forty-four of 104 cases (42.3%) were RUNX1–GLRX5 positive. Age, sex, smoking habit, EGFR mutations, histological type, and T factor were not significantly different between RUNX1–GLRX5-positive and -negative cases (Table 1). In addition, there were no significant differences in the mRNA expression level of RUNX1 and GLRX5 between both groups (unpaired *t*-test: $P = 0.486$ and $P = 0.805$, respectively).

Although the overall survival analysis did not show any significant differences between RUNX1–GLRX5-positive and -negative cases (log-rank test: $P = 0.088$; Fig. 1D), the relapse-free survival analysis

Table 1. Correlation between RUNX1–GLRX5 mRNA and clinicopathological features

	RUNX1–GLRX5 mRNA		P^a
	(−)	(+)	
Overall (n = 104)	60	44	
Year of age (mean ± SD)	67.0 ± 8.7	69.3 ± 10.1	0.230
Sex			
Male (n = 57)	35	22	
Female (n = 47)	25	22	0.399
Smoking habit			
Never (n = 41)	20	21	
Ever (n = 63)	40	23	0.138
EGFR mutation[b]			
Negative (n = 37)	20	17	
Positive (n = 28)	16	12	0.804
Histological type			
Adenocarcinoma (n = 88)	51	37	
Non-adenocarcinoma (n = 16)	9	7	0.899
T factor			
T1a (n = 78)	42	36	
T1b (n = 26)	18	8	0.169

[a]P values were calculated with the use of χ^2 test for numbers of categorical variables and unpaired t-test for year of age.

[b]The results of 65 cases in which the EGFR mutation status could be confirmed.

showed that the relapse rate was significantly lower in RUNX1–GLRX5-positive cases (log-rank test: $P = 0.039$; Fig. 1E). The first relapse sites in nine RUNX1-GLRX5-negative cases were the hilar lymph nodes, brain, lungs, chest wall, bone and adrenal gland, whereas the relapse site in 1 RUNX1–GLRX5-positive case was the lungs.

To evaluate the contribution of the expression level of RUNX1 and GLRX5 on the relapse-free period, a Cox proportional hazards analysis was performed. There was no significant difference in contribution to the relapse-free period for either gene (RUNX1: $P = 0.6$, GLRX5: $P = 0.117$).

Discussion

Stage IA NSCLC patients have been recognized to have a low risk of relapse, but the actual number of relapse cases is not small. We identified a novel chimeric transcript, RUNX1–GLRX5, and found that the relapse rate of RUNX1–GLRX5 -positive cases was significantly lower in Stage IA NSCLC patients, suggesting that this chimera can be a biomarker for good prognosis in Stage IA patients. Identifying Stage IA NSCLC patients who have a high relapse risk immediately after surgery would allow them to receive more frequent postoperative follow-up or adjuvant therapy.

We observed that the detection of RUNX1–GLRX5 was associated with a good prognosis, but this finding was unexpected because genetic abnormalities in solid tumors are generally associated with a poor prognosis. Indeed, we used chimera detection software (15) to search the publically available RNA-sequence data in NSCLC (16) and found that numbers of chimeric transcripts were associated with lymph node metastasis and smoking status (data not shown). The correlation between the presence of a specific chimera and a good prognosis remains difficult to explain. A thorough examination of the transcript expression level in RUNX1–GLRX5-positive and -negative cases may provide important information regarding this correlation.

There were no significant differences in the expression levels of RUNX1 and GLRX5 between RUNX1–GLRX5-positive and -negative cases. In addition, the contribution of RUNXI and GLRX5 expression on the relapse-free period was not significant. Thus, the expression levels of RUNX1 or GLRX5 cannot substitute for the detection of the chimeric transcript.

According to a report in which prostate cancer was studied, most frequently observed chimeras were fused between two parts of the same chromosome, and fusion between different chromosomes was rarely observed at a frequency of 50% or more (17,18). However, for RUNX1–GLRX5, although they are located on different chromosomes (21 and 14, respectively), ~50% of the Stage IA NSCLC cases that we investigated were positive for this chimeric transcript. It has been shown that the presence of a long intron promotes *trans*-splicing (19). Intron 2 of RUNX1, where the fusion is assumed to occur, is very long, comprising 155 878 bases. In addition, the presence of sequences shared by two introns may also promote *trans*-splicing (20,21). Several *Alu* sequences (22) are present in the sense and antisense chains in intron 2 of RUNX1, and one is present in the antisense chain in intron 1 of GLRX5. These may explain the high frequency of RUNX1–GLRX5 fusion. We consider that the RUNX1–GLRX5 fusion was not created by genomic rearrangement, but by an alteration at the transcriptome level, such as *trans*-splicing.

The pathophysiological activity of RUNX1–GLRX5 is unknown. We force-expressed RUNX1–GLRX5 in a lung cancer cell line, but a significant change in growth rate was not observed. In this study, we did not perform further functional analysis, such as migration assays, because we focused on its application as a clinical biomarker. In future, including a transcriptome analysis to detect any change in whole transcripts between RUNX1–GLRX5-positive and -negative cases will help further understanding of how RUNX1–GLRX5 contributes to a positive prognosis.

In conclusion, we successfully identified a new chimeric transcript, RUNX1–GLRX5, which can serve as a biomarker for good prognosis in Stage IA NSCLC.

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Conflict of interest statement

None declared.

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