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Detection of novel paraja ring finger 2-fer tyrosine kinase mRNA chimeras is associated with poor postoperative prognosis in non-small cell lung cancer

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Previously, we reported that the overexpression of fer tyrosine kinase (FER), a non-receptor tyrosine kinase, is correlated with poor postoperative prognosis and cancer-cell survival in non-small cell lung cancer (NSCLC). In the present study, we further analyzed FER-overexpressed NSCLC cases and identified various patterns of chimeric mRNAs, composed of paraja ring finger 2 (PJA2) and FER. We detected no genomic rearrangements between PJA2 and FER and attributed these chimeric mRNAs to alterations at the transcriptome level: i.e., *trans*-splicing. Several chimeric patterns were detected concurrently in each patient, and the pattern sets varied among patients, although the pattern in which PJA2 exon 1 was fused to FER exon 3 (designated as Pe1-Fe3 mRNA) was detected constantly. Therefore, in a wide screening for PJA2-FER mRNAs in NSCLC, we focused on this chimeric pattern as a representative chimera. In analyses of 167 NSCLC samples, Pe1-Fe3 mRNA was identified in about 10% of the patients, and the presence of chimeric mRNA was significantly correlated with a high expression level of parental FER mRNA. Furthermore, we found that the detection of Pe1-Fe3 mRNA was correlated with poor postoperative survival periods in NSCLC, consistent with a previous finding in which FER overexpression was correlated with poor postoperative prognosis in NSCLC. This report is the first to suggest a correlation between chimeric mRNA and the expression level of parental mRNA. Furthermore, our findings may be clinically beneficial, suggesting that PJA2-FER mRNAs might serve as a novel prognostic biomarker in NSCLC. (*Cancer Sci* 2013; 104: 1447–1454)

Despite significant advances in diagnosis and treatment, lung cancer remains the leading cause of cancer-related mortality worldwide, with over 1 million deaths each year,[(1)] and the 5-year survival rate of lung cancer is below 20%.[(2)] The best chance of achieving long-term survival is in complete surgical resection; however, even among resected stage IA patients, approximately 30% succumb to their disease within 5 years.[(3)] Although some recent trials have demonstrated that adjuvant chemotherapies improve the 5-year survival rate of patients with non-small cell lung cancer (NSCLC) who undergo complete surgical resection,[(4–6)] clear evidence of a clinical benefit of adjuvant chemotherapy has not yet been obtained. Given this situation, the ability to predict the clinical outcome of lung cancer after surgical resection and to identify patients who are at a high risk of recurrence and might benefit from further therapy after surgical resection is important. Some clinicopathological factors, such as tumor size, preoperative serum CEA level, visceral pleural invasion, vascular vessel invasion, and histological grade, are well-known predictors of clinical outcome in NSCLC patients after surgical resection.[(7–11)] In addition to these clinicopathological factors, to improve the clinical outcome of NSCLC, molecular prognostic biomarkers that can serve as more selective and creditable markers are needed.

Recent advances in high-throughput sequencing technology have provided novel insights into genomics; one of these insights is the discovery of chimeric mRNAs that are not caused by chromosomal rearrangements, but by transcription-induced mechanisms such as *trans*-splicing.[(12)] *Trans*-splicing is a form of alternative splicing in which RNA exons from two separate primary transcripts are joined, and this form of alternative splicing is now thought to be more common than previously believed[(13–15)] especially in cancers,[(16–18)] where the quality control systems governing transcription are frequently disrupted. Although some mechanisms that cause *trans*-splicing have been suggested, such as the close proximity of nascent pre-mRNAs[(14)] or the presence of complementary sequences between them,[(19,20)] the exact cause and regulation of *trans*-splicing remains to be elucidated.

In the present study examining NSCLC specimens, we identified various chimeric mRNAs, composed of paraja ring finger 2 (PJA2) and fer tyrosine kinase (FER), that probably resulted from *trans*-splicing. In a wide screening of NSCLC specimens, we found that the presence of chimeric mRNAs was positively correlated with a high expression level of parental FER mRNA. We also found that the detection of chimeric mRNAs was correlated with poor postoperative survival periods in a manner that was independent of other clinicopathological factors, consistent with our previous finding that FER overexpression is correlated with poor postoperative prognosis in NSCLC.[(21)]

To the best of our knowledge, this report is the first to suggest a correlation between chimeric mRNA and the expression level of parental mRNA; furthermore, because the RT-PCR method used here to detect the chimeric mRNAs is a simple method that can be easily performed at most institutions, PJA2-FER mRNAs may serve as a convenient prognostic biomarker in NSCLC.

Materials and Methods

Lung cancer patients and samples. Clinical lung cancer samples were collected from patients who had undergone surgical resection at the University of Tokyo Hospital between June 2005 and September 2007. The patient samples included those used in our previous study[(21)] but some other patients, from whom only nucleic acids were available, were newly added in the present study. Informed consent was obtained from all the patients, and the study was approved by the Institutional Ethics

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Review Committee. The diagnoses were based on pathological evidence and were classified according to the TNM classification criteria.[22] To circumvent statistical disruption arising from the heterogeneity of significantly advanced diseases, patients whose tumors were pathologically confirmed as T3 or T4 were excluded.

For all the patients, the medical records were reviewed to extract data on the clinicopathological characteristics. The follow-up data after surgical resection were obtained at our outpatient department. The routine physical examination and the result of chest X-ray were recorded every 3 months in the first 5 years postoperatively, and twice a year thereafter. Chest computed tomography was performed every 6 months. Progression-free survival was measured from the date of surgery until disease recurrence, metastasis, or death, and overall survival was measured from the date of surgery until the date of death. Patients without a known date of death were censored at the time of the last follow-up.

Rapid amplification of cDNA ends. Rapid amplification of cDNA ends (RACE) products were obtained from clinical samples using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) and KOD -Plus- Ver. 2 (Toyobo, Osaka, Japan), a high-fidelity DNA polymerase with 3′ to 5′ exonuclease activity, according to the manufacturer's protocol. The 3′ end primer for 5′ RACE and the 5′ end primer for 3′ RACE, which were called Gene Specific Primer (GSP) 1 and 2 in the kits, respectively, of FER were designed as follows: GSP 1, 5′-GCT GAT TTG TCA TTC CAG GGT ACG G-3′; GSP 2, 5′-GAC AGC CTG TCT ACA TCA TTA TGG AAC-3′; nested GSP (NGSP) 1, 5′-GCT TTA AGC CAG AAG ATG AAT ACA CTC C-3′; and NGSP 2, 5′-CGC TGC TGC TGG TAT GTT GTA TCT C-3′. The RACE products were then sequenced using cycle sequencing on a Mastercycler (Eppendorf, Hamburg, Germany) with the BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and were analyzed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

PCR of genomic DNA to detect the genomic fusion point between PJA2 and FER. To detect the genomic fusion point between PJA2 and FER, multiple primers were designed at exon 7 and intron 7 of PJA2 in a downward direction and at exon 3 and intron 2 of FER in an upward direction (Table S1), targeting the site where the genomic rearrangement was estimated to occur based on the detected patterns of chimeric transcripts. Polymerase chain reaction was performed using all possible pairs of these primers under every possible condition using KOD -Plus- Ver. 2 (Toyobo), PrimeSTAR GXL DNA Polymerase (TaKaRa, Shiga, Japan), or Phusion High-Fidelity DNA Polymerase (New England Biosystems, Ipswich, MA, USA). Extracted DNA from lung cancer specimens was used as a template.

Genome-walking sequence. To sequence the PJA2 gene from the 5′ end to the 3′ end thoroughly and to detect the possible genomic fusion point between PJA2 and FER, the Genome-Walker Universal Kit (Clontech) was used according to the manufacturer's protocol. Briefly, the genomic DNA extracted from normal lung tissue and the PJA2-FER mRNA-positive lung cancer specimen were separately digested using four different restriction enzymes. The digested DNA fragments were then adaptor-ligated to produce four sets of DNA fragments with adapters at their ends. After the four libraries were constructed, PCR and nested PCR amplifications of each library were conducted between Adaptor Primer (provided by the manufacturer) and primers specific to PJA2: 5′-TAT TTG ATG GCT TTG CAG ATG GAC TAG GAG-3′ as gene-specific primer 1 and 5′-CCT ACA TGG CAC TAG AAG AAC GCT TAG-3′ as gene-specific primer 2. The amplifications were performed using PrimeSTAR GXL DNA Polymerase (TaKaRa). All the PCR products were TA cloned and subsequently sequenced in the same manner as described above.

Quantitative RT-PCR. The expression levels of PJA2 and FER mRNA in normal human tissues and lung cancer specimens were examined using quantitative RT-PCR. The total RNA from lung cancer specimens was isolated using RNAiso (TaKaRa), and the FirstChoice Human Total RNA Survey Panel (Applied Biosystems) was used as RNA from normal human tissues. The RNA products were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). SYBR green RT-PCR was performed using Thunderbird qPCR Mix (Toyobo) and was analyzed using Applied Biosystems 7500, according to the manufacturer's instructions. The following primers were used: 5′-AAG GCA AGA GGT GGA TAA CAC-3′/5′-AGG GCC ACT GCT ATC ACT T-3′ for PJA2, and 5′-ACT GCA CGT TTT CTC AAG TAT CTT-3′/5′-CTT ACT ACT GTT CAT TGC TGG-3′ for FER. Relative expression level of FER mRNA was calculated using the $\Delta\Delta C_t$ method with the β-actin gene as an internal control, and a level more than 16 times higher than the expression level in normal lung tissue was set as the threshold for FER mRNA overexpression.

Immunohistochemistry. The FER immunohistochemistry (IHC) score data of clinical samples analyzed in the previous study[21] were used in the present study.

RT-PCR to detect chimeric PJA2-FER mRNAs. PJA2-FER mRNAs were detected using RT-PCR methods using cDNA as a template. Amplification was performed using AmpliTaq Gold 360 Master Mix (Applied Biosystems). To detect all the possible chimeric mRNAs, the top primer was designed at exon 1 of PJA2 in a downward direction (5′-GCG GAG AAG AAG GCG GTG GT-3′) and the bottom primer was designed at exon 19 of FER in an upward direction (5′-GCT GAT TTG TCA TTC CAG GGT ACG G-3′). To detect the chimeric pattern in which exon 1 of PJA2 was fused to exon 3 of FER, the top primer was designed at exon 1 of PJA2 in a downward direction (5′-AGG TTC GAG CGC TGT TCT C-3′) and the bottom primer was designed at exon 3 of FER in an upward direction (5′- CCG TAA TTC CCA GTC TTG CAA T-3′). The resulting PCR products were sequenced in the same manner as described above.

Plasmid vector constructions. PJA2-FER cDNAs were cloned using a previously reported method[23] and ligated into the NotI restriction sites of the mammalian expression vector pcDNA 3.1 Hygro$^+$ (Invitrogen). The final products were sequenced in the same manner as described above to confirm the absence of unexpected mutations.

Focus formation assay. NIH-3T3 cells were transfected using PJA2-FER expression plasmid vectors and HilyMax (Dojindo, Kumamoto, Japan). The cells were cultivated in the presence of hygromycin to select the transfected cells, and whether the surviving cells formed foci after reaching confluence was noted. Pictures were taken after 14 days of culture.

Colony formation assay. A total of 1000 cells were seeded onto Hydrocell (Cell Seed, Tokyo, Japan), which are non-adhesive dishes coated with hydrophilic polymers, in a normal medium containing 1.3% methylcellulose (Wako, Osaka, Japan). After incubation for 3 days, the formed colonies were quantified using the Cell Counting Kit-8 assay (Dojindo). Viable cells forming colonies were evaluated based on absorbance measurements performed at 450 nm using a 1420 Multilabel Counter (Perkin Elmer Life Sciences, Boston, MA, USA).

Statistical analysis. A statistical analysis was performed using DR. SPSS II (SPSS Inc., Chicago, IL, USA). To analyze the differences in the FER mRNA expression levels between PJA2-FER mRNA-positive cases and PJA2-FER mRNA-negative cases, the Student *t*-test was used. The relation between the detection of PJA2-FER mRNA and FER overexpression was analyzed using

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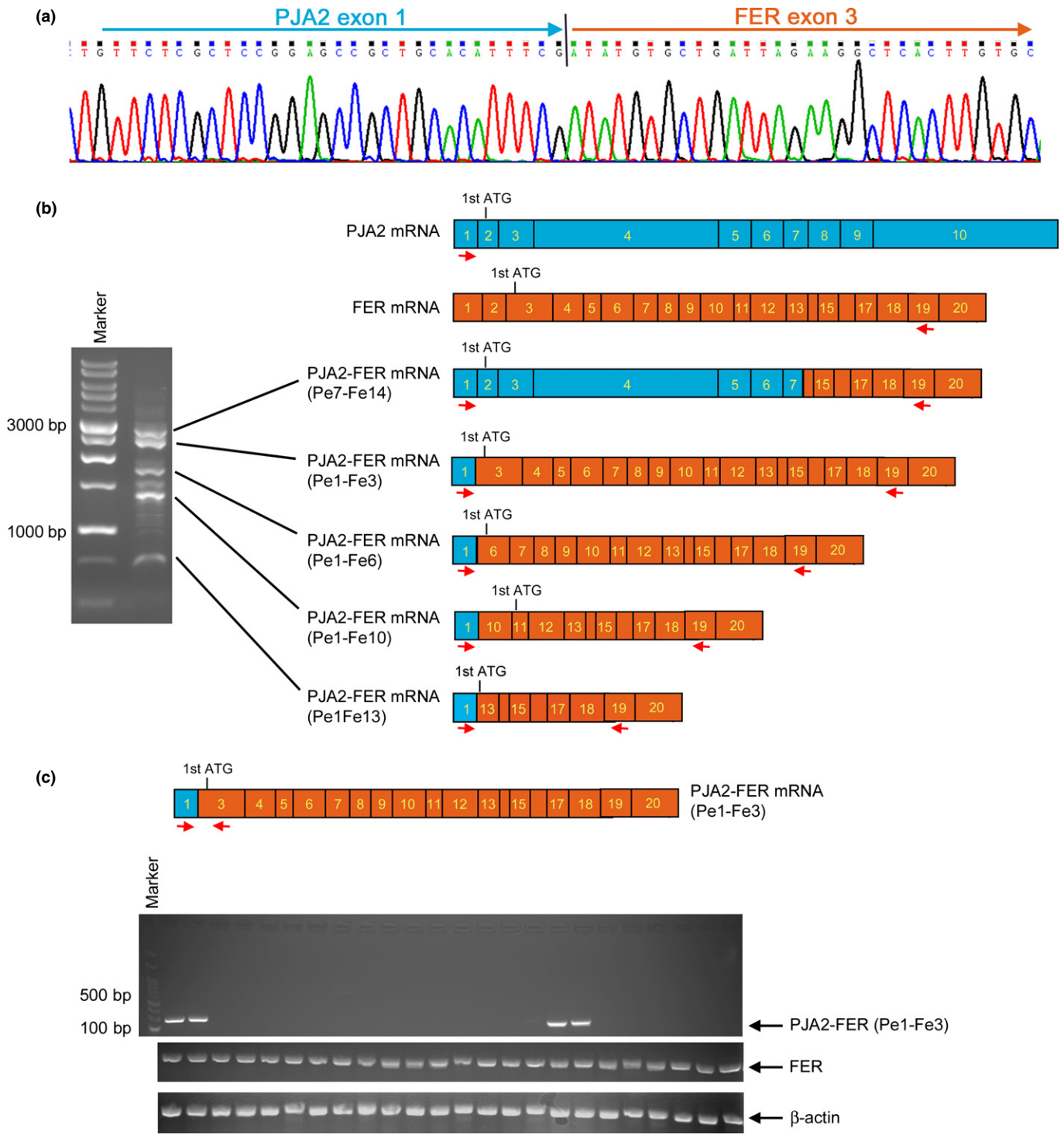

Fig. 1. Identification of chimeric PJA2-FER mRNAs. (a) Sequence chromatogram showing exon 1 of PJA2 mRNA fused to exon 3 of FER mRNA. (b) RT-PCR to detect chimeric PJA2-FER mRNAs. An image of the RT-PCR products on an agarose gel and a schema of the corresponding PJA2-FER mRNAs are shown. The red arrows represent the primers used to detect PJA2-FER mRNAs. The number indicates the exon number, and the exons from PJA2 and FER are shown in blue and red, respectively. Marker, 1000-bp DNA ladder. (c) Wide screening for Pe1-Fe3 mRNA in non-small cell lung cancer (NSCLC) samples. A representative image of the RT-PCR products on an agarose gel is shown. The red arrows represent the primers used to detect Pe1-Fe3 mRNA. The number indicates the exon number, and the exons from PJA2 and FER are shown in blue and red, respectively. Marker, 100-bp DNA ladder.

the Fisher's exact test. To analyze the differences in the colony formation assay, the Tukey's honestly significant differences test was used. The relation between PJA2-FER mRNA and the clinicopathological characteristics of NSCLC was analyzed using the χ^2 test or the Fisher's exact test, and a logistic regression analysis was used to analyze independent factors associated with PJA2-FER mRNA. The progression-free and overall survival curves were calculated using the Kaplan–Meier method and

were compared using a log-rank test. A multivariate Cox regression analysis of factors was performed to identify independent factors related to the prognosis of NSCLC patients.

Results

Identification of chimeric PJA2-FER mRNAs. To analyze the genetic events related to FER-overexpressed NSCLC, we performed RACE using cDNA from a 67-year-old female patient with FER overexpression. We detected several RACE products, one of which was confirmed to be an intact-type of FER mRNA after sequencing (data not shown). When we sequenced other RACE products, most of them were identified as non-specific products unrelated to FER; however, surprisingly, we found that one of them appeared to be a chimeric mRNA (DDBJ/EMBL/GenBank Accession No. AB636592), in which the 3′ side downstream from exon 3 of FER was fused to exon 1 of PJA2 (Fig. 1a).

Considering the possibility of other chimeric patterns between PJA2 and FER, we decided to perform RT-PCR using a top primer designed at exon 1 of PJA2 in a downward direction and a bottom primer designed at exon 19 of FER in an upward direction. When we performed RT-PCR using cDNA from the same patient, a number of RT-PCR products were obtained; by sequencing these products, we found various patterns for chimeric PJA2-FER mRNAs (DDBJ/EMBL/GenBank Accession Nos. AB636592–AB636596) (Fig. 1b). We designated these patterns according to the exon number of the fused location, such as Pe7-Fe14 mRNA (in which exon 7 of PJA2 was fused to exon 14 of FER) or Pe1-Fe3 mRNA (in which exon 1 of PJA2 was fused to exon 3 of FER) (Fig. 1b). All the chimeric mRNAs detected here contained an open reading frame with the first ATG shown in Figure 1(b), but unfortunately, we could not find a suitable antibody for the N terminus of PJA2 or the C terminus of FER to examine the translation of the chimeric proteins or truncated FER proteins that were predicted to be translated from these chimeric mRNAs.

Next, we attempted to find the genomic fusion point by performing PCR using the genomic DNA as a template. PJA2 and FER both map to the long arm of chromosome 5 (5q21) but have opposite orientations and are separated by a distance of 147 kilobases. Considering the patterns of the detected chimeric mRNAs, the disrupted and ligated position in the DNA was estimated to be downstream of PJA2's exon 7 and upstream of FER's exon 3. Therefore, we designed multiple top primers at exon 7 and intron 7 of PJA2 in a downward direction and multiple bottom primers at exon 3 and intron 2 of FER in an upward direction and then performed multiple PCR analyses by pairing all these primers under every possible PCR condition. However, no significant PCR products were identified (data not shown). We also attempted to detect the possible genomic fusion point by thoroughly sequencing the PJA2 gene from the 5′ end to the 3′ end. To identify the fused point, we used genome-walking methods. We ligated the adaptor to genomic DNA after digestion with multiple restriction enzymes and performed PCR with primers specific to the adaptor and PJA2, followed by the sequencing of all the products with primer walking; however, all the detected bands were derived from intact-type PJA2, and no genomic rearrangement was detected (data not shown). These results suggest that chimeric PJA2-FER transcripts are attributed not to genomic rearrangement, but to an alteration at the transcriptome level: i.e., *trans*-splicing.

Correlation between PJA2-FER mRNA and expression level of FER mRNA. When a few other FER-overexpressed NSCLC specimens were examined, we detected more various chimeric PJA2-FER mRNAs in several cases with different sets of patterns (Table 1). We decided to perform a wide screening for PJA2-FER mRNAs in a broader population of NSCLC patients including those without FER overexpression; however, the detection of all possible chimeric patterns, which have a wide variety, was thought to be cumbersome. Among the various detected chimeric patterns, the Pe1-Fe3 pattern, in which exon 1 of PJA2 was fused to exon 3 of FER, was constantly detected (Table 1). Therefore, we decided to focus on this chimeric pattern and to use this pattern as a representative of PJA2-FER mRNAs in a wide screening.

We designed a new top primer within exon 1 of PJA2 and a new bottom primer within exon 3 of FER and performed RT-PCR to detect chimeric Pe1-Fe3 mRNA as a screening for PJA2-FER mRNAs in NSCLC specimens (Fig. 1c). The RT-PCR products were sequenced, and we confirmed that these products represented Pe1-Fe3 mRNA. As a result, chimeric mRNA was identified in 13.2% (17/128 cases) of the lung adenocarcinoma cases and 7.7% (3/39 cases) of the squamous cell lung cancer cases. In addition, FER itself was detected in all the specimens, and FER overexpression could not be distinguished using this simple RT-PCR method (Fig. 1c).

Next, to search for contributing factors to the generation of chimeric mRNAs, we decided to examine the correlation between the chimeric mRNAs and the expression level of parental mRNA, assuming that a larger amount of material (parental pre-mRNA) would produce products (chimeric mRNAs) more easily. In the quantitative RT-PCR analysis, the expression levels of PJA2 mRNA were moderately high, while the expression levels of FER mRNA were relatively low, in normal human tissues ubiquitously (Fig. 2a). When we

Table 1. Detected patterns of chimeric PJA2-FER mRNAs in FER-overexpressed non-small cell lung cancer (NSCLC) cases

Case no.	Patterns of chimeric PJA2-FER mRNAs							
	Pe7-Fe14	Pe1-Fe3	Pe1-Fe4	Pe1-Fe6	Pe1-Fe10	Pe1-Fe13	Pe1-Fe14	Pe1-Fe17
1	†	†	—	†	†	†	—	—
2	—	†	—	—	—	†	—	—
3	—	†	—	†	†	—	—	—
4	—	†	—	—	—	—	†	—
5	†	†	—	—	—	—	—	—
6	—	†	†	—	†	—	—	—
7	†	†	—	—	†	—	—	—
8	—	†	—	—	—	—	—	†
9	—	†	—	†	—	†	—	—
10	†	†	—	†	—	—	—	†

Chimeric patterns were designated according to the exon number of the fused location as described in the text. †Indicates that the chimeric pattern was detected in the case.

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examined NSCLC samples, we found that while the expression level of PJA2 mRNA was constantly highly expressed as observed in normal human lung tissue, the expression level of FER mRNA varied among the cases from less than a hundredth to the same level of PJA2 mRNA expression (Fig. 2b). The primers used here in the quantitative RT-PCR analysis to examine the expression level of FER mRNA were designed within exon 2 of FER, which is located upstream of the detected chimeric points; therefore, the expression level of FER mRNA examined here should reflect the expression level of intact FER mRNA (but not PJ2-FER mRNA).

We assumed that, because the PJA2 mRNA expression level is invariably high, the FER mRNA expression level might have a decisive influence on the generation of PJA2-FER mRNAs in NSCLC. As expected, when we examined the correlation between the presence of Pe1-Fe3 mRNA and the expression level of FER mRNA, we found a significant positive correlation (Fig. 2c, Table 2), supporting our hypothesis that larger quantities of parental FER pre-mRNAs are more likely to form chimeric mRNAs with PJA2 pre-mRNAs. This positive correlation was verified even when we analyzed the association between FER mRNA expression level examined with the primer set used in the previous study[21] and Pe1-Fe3 mRNA status (Table S2). We also confirmed that Pe1-Fe3 mRNA status was correlated with immunostaining score for FER (Table S3).

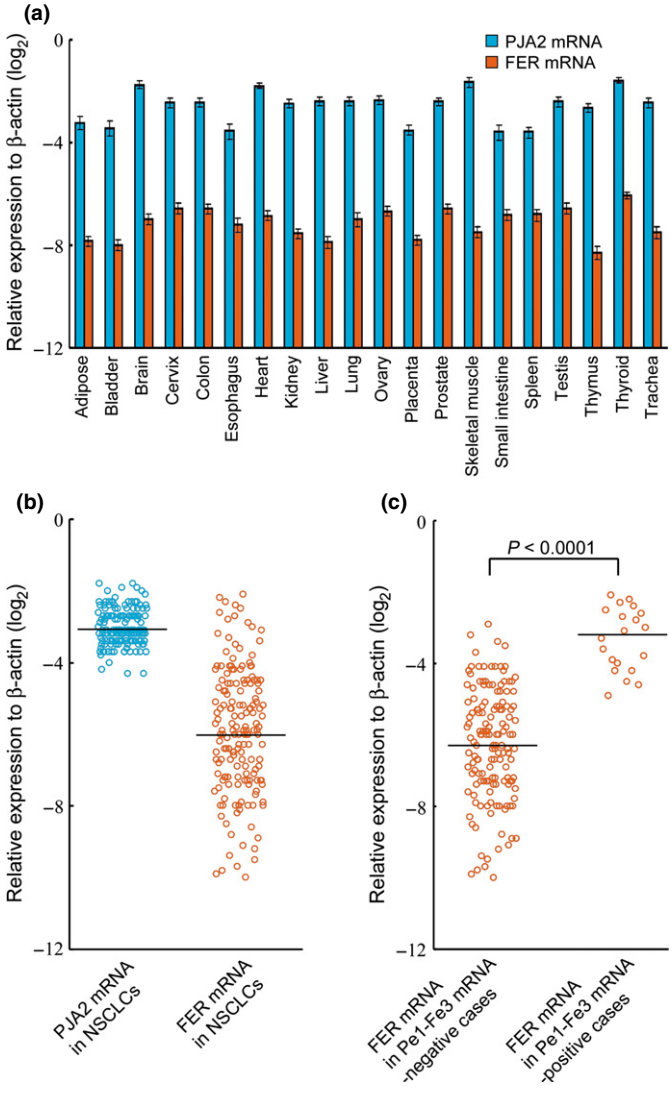

Fig. 2. Expression levels of PJA2 and FER mRNA. (a) Expression level of PJA2 mRNA and FER mRNA in several normal human tissues. The error bars indicate the standard deviations. (b) Comparison of the expression levels between PJA2 mRNA and FER mRNA in NSCLC cases. Each circle indicates data obtained from each specimen. Data for PJA2 mRNA and FER mRNA are shown in blue and red, respectively. The bars indicate the median values. (c) Comparison of the FER mRNA expression level between Pe1-Fe3 mRNA-positive cases and Pe1-Fe3 mRNA-negative cases. Each circle indicates data obtained from each specimen. The bars indicate the median values.

Table 2. Correlation between detection of Pe1-Fe3 mRNA and FER mRNA overexpression

	Pe1-Fe3 mRNA (+)	Pe1-Fe3 mRNA (−)
FER mRNA overexpression (+), *n*	19	3
FER mRNA overexpression (−), *n*	1	144
Total, *n*	20	147

Fisher's exact test $P < 0.001$.

Biological function of chimeric PJA2-FER mRNAs. Although whether novel proteins are translated from chimeric PJA2-FER mRNAs could not be examined because of the absence of a suitable detection antibody as mentioned above, we decided to examine whether PJA2-FER chimeras have any biological functions *in vitro*. We constructed their expression plasmid

Table 3. Baseline characteristics of 167 analyzed non-small cell lung cancer (NSCLC) cases

Characteristics	Patient cohort (*n* = 167)
Age	
Median, years	69
Interquartile range, years	34–85
≥65 years, *n* (%)	98 (58.7)
<65 years, *n* (%)	69 (41.3)
Sex	
Male, *n* (%)	101 (60.5)
Female, *n* (%)	66 (39.5)
Smoking habit	
Never, *n* (%)	40 (24.0)
Ever, *n* (%)	127 (76.0)
Histological type	
Adenocarcinoma, *n* (%)	128 (76.6)
Squamous cell carcinoma, *n* (%)	39 (23.4)
Disease stage	
IA, *n* (%)	71 (42.5)
IB, *n* (%)	44 (26.3)
IIA, *n* (%)	7 (4.2)
IIB, *n* (%)	18 (10.8)
IIIA, *n* (%)	27 (16.2)
T factor	
T1, *n* (%)	84 (50.3)
T2, *n* (%)	83 (49.7)
N factor	
N0, *n* (%)	122 (73.0)
N1, *n* (%)	21 (12.6)
N2, *n* (%)	24 (14.4)
Adjuvant chemotherapy	
Yes, *n* (%)	41 (24.6)
No, *n* (%)	126 (75.4)
EGFR mutation	
Negative, *n* (%)	107 (64.1)
Positive, *n* (%)	50 (29.9)
Unknown, *n* (%)	10 (6.0)

Table 4. Correlation between Pe1-Fe3 mRNA and clinicopathological features

	Pe1-Fe3 mRNA		*P*
	(+), *n*	(−), *n*	
Overall (*n* = 167)	20	147	
Age			
≥65 years (*n* = 98)	11	87	
<65 years (*n* = 69)	9	60	0.810
Sex			
Male (*n* = 101)	14	87	
Female (*n* = 66)	6	60	0.467
Smoking habit			
Never (*n* = 40)	5	35	
Ever (*n* = 127)	15	112	1.000
Histological type			
Adenocarcinoma (*n* = 128)	16	112	
Squamous cell carcinoma (*n* = 39)	4	35	1.000
Disease stage			
IA, IB (*n* = 115)	13	102	
IIA-IIIA (*n* = 52)	7	45	0.797
T factor			
T1 (*n* = 84)	10	74	
T2 (*n* = 83)	10	73	1.000
N factor			
N0 (*n* = 122)	15	107	
N1, N2 (*n* = 45)	5	40	1.000
Adjuvant chemotherapy			
Yes (*n* = 41)	4	37	
No (*n* = 126)	16	110	0.784
EGFR mutation			
Negative (*n* = 107)	14	93	
Positive (*n* = 50)	6	44	1.000

vectors and introduced them into NIH-3T3 cells. When we performed assays to examine oncogenic functions *in vitro*, such as a focus formation assay to assess the loss of contact inhibition and a colony formation assay to assess the anchorage independence of cell growth, mutant KRAS and intact FER, which have been shown to harbor transforming activity,[21] were confirmed to have oncogenic functions; in contrast, however, the PJA2-FER chimeras that we examined showed no transforming activities as well as a negative control (GFP) (Fig. S1).

Correlation between PJA2-FER mRNA and postoperative survival periods in NSCLC patients. As the FER expression level was previously shown to be correlated with poor postoperative prognosis in NSCLC,[21] we were motivated to investigate whether the detection of PJA2-FER mRNA, which was shown to be correlated with a high expression level of FER, was also correlated with the clinical outcome in NSCLC. To this end, we analyzed the clinical data of 167 patients with primary NSCLC who had undergone a potential curative resection. The baseline characteristics of the patient cohort are shown in Table 3. The median follow-up period was 59.2 months.

When we analyzed the correlations between the detection of Pe1-Fe3 mRNA and various clinicopathological features, such as age, sex, smoking history, histological type, disease stage, T factor, N factor, adjuvant chemotherapy history, or EGFR mutation status, no significant correlations were observed in univariate analyses (Table 4). However, in a Kaplan–Meier analysis, we found that the detection of Pe1-Fe3 mRNA was significantly associated with poor progression-free and overall survival periods after surgical resection (Fig. 3). To control for potential confounding effects of other factors, we further performed a multivariate Cox regression analysis and found that Pe1-Fe3 mRNA was an independent prognostic factor for both progression-free and overall survival periods in addition to age, disease stage, T factor and N factor (Table 5).

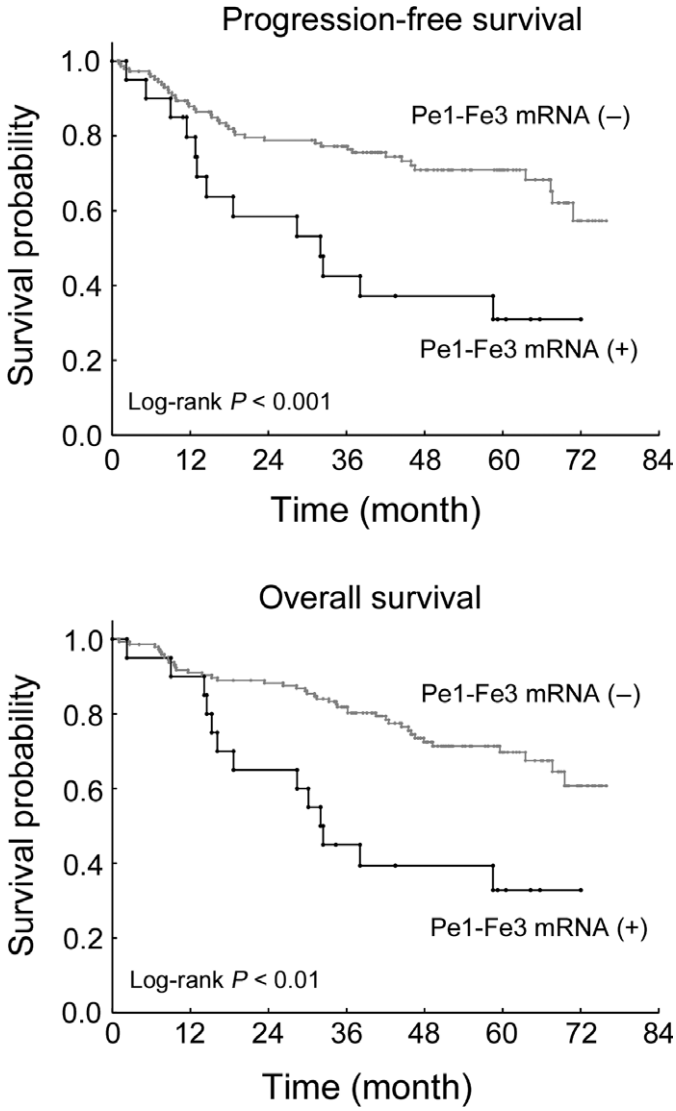

Fig. 3. Correlation between the detection of PJA2-FER mRNA and the postoperative prognosis of non-small cell lung cancer (NSCLC) patients. Kaplan–Meier analyses of the progression-free and overall survival periods among 167 curatively resected NSCLC patients are shown stratified according to the detection of Pe1-Fe3 mRNA.

Discussion

Here, we identified novel chimeric PJA2-FER mRNAs that were correlated with a high expression level of parental FER mRNA and served as a potential biomarker for the postoperative prognosis in NSCLC. PJA2 and FER are located next to each other, but in inverse orientations, on chromosome 5q. PJA2 encodes an E3 ubiquitin-protein ligase, and although the precise function of PJA2 has not been clarified, its expression level is moderately high in almost all normal tissues according to our present quantitative RT-PCR results and microarray analysis (RefExA, http://www.lsbm.org/). FER is a 94-kDa non-receptor tyrosine kinase involved in the reorganization of the actin cytoskeleton or the modulation of cell–cell and cell–substrate interactions through

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Table 5. Multivariate Cox regression analysis of various factors for prognosis of lung cancer patients

Variables	Hazard ratio (95% CI)	Unfavorable/favorable	*P*
Progression-free survival			
Pe1-Fe3 mRNA	2.997 (1.472–6.100)	Positive/negative	0.002*
Age (years)	3.050 (1.097–8.482)	≥65/<65	0.033*
Sex	1.672 (0.888–3.148)	Male/female	0.112
Smoking habit	1.550 (0.564–4.260)	Ever/never	0.395
Histological type	1.047 (0.425–2.579)	Adeno/squamous	0.921
Disease stage	3.905 (1.684–9.056)	IIA–IIIA/IA, IB	0.002*
T factor	4.310 (2.206–8.419)	T2/T1	0.001*
N factor	1.893 (0.725–4.941)	N1, N2/N0	0.049*
Adjuvant chemotherapy	1.511 (0.769–2.970)	No/yes	0.231
EGFR mutation	1.195 (0.625–2.284)	Negative/positive	0.589
Overall survival			
Pe1-Fe3 mRNA	3.313 (1.594–6.888)	Positive/negative	0.001*
Age (years)	3.332 (1.235–8.991)	≥65/<65	0.017*
Sex	1.736 (0.934–3.228)	Male/female	0.081
Smoking habit	2.045 (0.750–5.572)	Ever/never	0.162
Histological type	1.367 (0.537–3.483)	Adeno/squamous	0.512
Disease stage	4.062 (1.714–9.628)	IIA–IIIA/IA, IB	0.001*
T factor	3.502 (1.791–6.846)	T2/T1	0.001*
N factor	2.098 (0.798–5.520)	N1, N2/N0	0.048*
Adjuvant chemotherapy	1.551 (0.787–3.057)	No/yes	0.205
EGFR mutation	1.012 (0.529–1.936)	Negative/positive	0.972

*$P < 0.05$. CI, confidence interval.

its association with adhesion molecules[24–31] and has been reported to play a role in the proliferation and growth of several cancers,[32] such as prostate cancer,[33,34] colon cancer,[35] breast cancer,[36] hepatocellular carcinoma,[37] malignant mesothelioma,[38] and gastric cancer.[39] Especially, very recently, FER expression has been shown to be correlated with poor prognosis in NSCLC[21] and renal cell carcinoma.[40,41]

We attempted the PCR amplification of a putative recombined chromosome using several primers flanking the expected translocation site, but no genomic PJA2-FER fusion sequences were detected. Thus, we attributed these chimeric mRNAs to alterations at the transcriptome level: i.e., *trans*-splicing. *Trans*-splicing is a form of alternative splicing in which mRNA exons from two separate primary transcripts are joined. Since this phenomenon was first discovered in trypanosomes,[42] several findings have demonstrated that it may also occur in mammalian cells, and this form of alternative splicing has now been shown to be more common than previously believed.[13–15] As for the mechanisms that cause *trans*-splicing, some reports suggest that *trans*-splicing occurs at high frequencies when the parental pre-mRNAs are closely positioned to each other or when there are complementary sequences between them.[14,19,20,43]

In the case of PJA2 and FER, because the PJA2 gene is located next to the FER gene on the same chromosome, the two pre-mRNAs are thought to be present in very close proximity to each other, possibly creating a predisposition toward *trans*-splicing between them. In addition, interestingly, we found that there was a significant positive correlation between the presence of Pe1-Fe3 mRNA and a high expression level of parental FER mRNA in the present study. From a biochemical perspective, it seems reasonable to assume that a larger amount of material (pre-mRNAs) would be likely to produce products (chimeric transcripts) more easily and that an abundance of parental transcripts might be an accelerating factor contributing to *trans*-splicing. As for PJA2 and FER, because the expression level of PJA2 mRNA is invariably high among NSCLC cases, the likelihood of *trans*-splicing between them would depend on the expression level of FER mRNA.

Regarding biological function, *trans*-splicing is generally expected to increase proteomic diversity, but its exact significance remains controversial. In cancer, some *trans*-splicing products, such as CYCLIN D1-TRO2 mRNA in ovarian and mammary cancer[44] or SLC45A3-ELK4 mRNA in prostate cancer,[17,45] are reported to be involved in the oncogenic mechanism. In our study, we could not examine whether novel proteins were translated from chimeric PJA2-FER mRNAs, and as far as we were able to examine using *in vitro* assays, the PJA2-FER chimeras showed no transforming activities. Additionally, when we analyzed the amino acid sequences that were possibly translated from PJA2-FER mRNAs *in silico*, they were not predicted to have any domain that might be related to oncogenic function. However, further functional analyses are needed to examine whether they have any other biological functions.

On the other hand, because FER overexpression has been previously shown to be correlated with poor postoperative prognosis in NSCLC,[21] we assumed that PJA2-FER mRNA, which was found to be correlated with a high expression level of FER mRNA, might also serve as a prognostic biomarker in NSCLC. As expected, the detection of Pe1-Fe3 mRNA was shown to be correlated with poor postoperative prognosis in patients with NSCLC independently of other clinical prognostic factors, although the correlation with further factors such as histological subtype, which is an established and convenient prognostic factor for stage I adenocarcinoma, should be analyzed in the future studies with a larger number of samples.

Reverse transcription-polymerase chain reaction was used in the present study to detect Pe1-Fe3 mRNA, and this method is relatively simple and can easily be performed at most institutions. As shown in Figure 1(c), FER itself was detected as a band using RT-PCR method in all the NSCLC samples, including those without FER overexpression. Therefore, to distinguish FER expression level, simple RT-PCR method is not sufficient, and further quantitative RT-PCR or IHC analyses are needed, which are sometimes difficult to perform and lead to controversial results because of technical inadequacies or cost problems in some institutions or because of the inadequate volume of resected samples. Considering such situations, the identification of Pe1-Fe3 mRNA as a biomarker that can be examined easily as the detection of a band using a simple RT-PCR method is likely to be clinically more beneficial.

In conclusion, we have identified novel chimeric PJA2-FER mRNAs that were correlated with a high expression level of parental FER mRNA and poor postoperative prognosis in NSCLC. Although further molecular studies or a larger epidemic study are needed, our findings might provide some insights into the mechanism of *trans*-splicing and might also be clinically beneficial by helping to easily identify NSCLC patients at risk after surgery who are likely to benefit from adjuvant chemotherapy, thereby improving the clinical outcome of NSCLC.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Functional analyses of PJA2-FER chimeras.

Table S1. Primers used for detection of genomic fusion point between PJA2 and FER.

Table S2. Correlation between detection of Pe1-Fe3 mRNA and FER mRNA expression level examined with the primer set used in the previous study.

Table S3. Correlation between detection of Pe1-Fe3 mRNA and FER immunostaining score.

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