BRIEF REPORT

Brief Report: circRUNX1 as Potential Biomarker for Cancer Recurrence in EGFR Mutation-Positive Surgically Resected NSCLC

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ABSTRACT

Introduction: As recently evidenced by the ADAURA trial, most patients with stages IB to IIIA of resected EGFR-mutant lung adenocarcinoma benefit from osimertinib as adjuvant therapy. Nevertheless, predictive markers of response and recurrence are still an unmet need for more than 10% of these patients. Some circular RNAs (circRNAs) have been reported to play a role in tumor growth and proliferation. In this project, we studied circRNA expression levels in formalin-fixed, paraffin-embedded lung tumor samples to explore their biomarker potential and develop a machine learning (ML)-based signature that could predict the benefit of adjuvant EGFR tyrosine kinase inhibitors in patients with EGFR-mutant NSCLC.

Methods: Patients with surgically resected EGFR mutant-positive, stages I to IIIB NSCLC were recruited from February 2007 to December 2015. Formalin-fixed, paraffin-embedded tumor samples were retrospectively collected from those patients with a follow-up period of more than or equal to 36 months (N = 76). Clinicopathologic features were annotated. Total RNA was purified and quantified prior nCounter processing with our circRNA custom panel. Data analysis and ML were performed taking into consideration circRNA expression levels and recurrence-free survival (RFS). RFS was defined from the day of surgery to the day when recurrence was detected radiologically or the death owing to any cause.

Results: Of the 76 patients with EGFR mutation-positive NSCLC included in the study, 34 relapsed within 3 years after resection. The median age of the relapsing cohort was 71.5 (range: 49–89) years. Most patients were nonsmokers (n = 21; 61.8%) and of female sex (n = 21; 61.8%). Most cases (n = 17; 50%) presented an exon 21 mutation, whereas 15 and four patients had an exon 19 and exon 18 mutation, respectively. Differential expression analysis revealed that circRUNX1, along with circFUT8 and circAASDH, was up-regulated in relapsing patients ($p < 0.05$ and >2 fold-change). A ML-based circRNA signature predictive of recurrence in patients with EGFR mutation-positive NSCLC, comprising circRUNX1, was developed. Our final model including selected 6-circRNA signature with random forest algorithm was able to classify relapsing patients with an accuracy of 83% and an area under the receiver operating characteristic curve of 0.91. RFS was significantly shorter not only for the subgroup of patients with high versus low circRUNX1 expression but also for the group classified as recurrent by the ML circRNA signature when compared with those classified as nonrecurrent.

Conclusions: Our findings suggest that circRUNX1 and the presented ML-developed signature could be novel tools to predict the benefit of adjuvant EGFR tyrosine kinase

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inhibitors with regard to RFS in patients with EGFR-mutant NSCLC. The training and validation phases of our ML signature will be conducted including bigger independent cohorts.

Keywords: CircRUNX1; Biomarker; EGFR mutation; NSCLC

Introduction

The 5-year survival rate for patients with stages IB to III resected EGFR-mutant NSCLC has been reported as 88% with adjuvant osimertinib therapy for Asian patients, compared with 78% in the placebo arm (hazard ratio [HR] for death = 0.49, $p < 0.001$), on the basis of findings from ADAURA trial.[1] The differences were not so consistent according to mutation type (ex19 del versus L858R) and race (Asian versus non-Asian). Moreover, the benefit in terms of disease-free survival (DFS) was less prominent in patients with L858R mutation, Asian patients, and those with stage IB.[2] Likewise, similar results were found by the subgroup analysis from the FLAURA trial in EGFR-mutant metastatic NSCLC comparing first-line osimertinib therapy versus gefitinib or erlotinib.[3]

L858R mutations are more susceptible to harbor TP53 mutations and 3q amplification and to be T790M negative in comparison with ex19 del EGFR-mutated NSCLC.[4] Besides all efforts on studying the different molecular mechanisms underlying adjuvant tyrosine kinase inhibitor resistance in resected EGFR-mutant NSCLC, the identification of predictive markers of response and recurrence is still an unmet need in the clinical setting.

Circular RNAs (circRNAs) are a type of single-stranded RNA involved in the pathogenesis of lung cancers.[5]

With a complex covalently closed structure, these molecules stand out as a novel class of lung cancer biomarkers, providing additional information to that from their linear counterparts owing to their different biological functions, including microRNA (miRNA) sponging. In addition, their circular architecture provides robustness to these molecules, which along with their tissue-specific expression makes them perfect markers for diagnosis and prognosis. Here, we report our results on the study of circRNA expression patterns and machine learning (ML) application for the identification of new biomarkers of recurrence in patients with surgically resected EGFR-mutant NSCLC.

Materials and Methods

Surgical specimens were collected from patients with primary invasive resected NSCLC between February 2007 and December 2015 at the Hiroshima University Hospital, Japan. Patients, after preoperative chemotherapy, chemoradiotherapy, or incomplete resection, or with unavailable specimens or clinicopathologic data, were excluded from the study. Patients with adenocarcinoma in situ and minimally invasive adenocarcinoma were also excluded, owing to no recurrence after complete resection. Finally, those patients whose follow-up was less than 36 months were also excluded from the study. Written informed consent was obtained from all patients and further documented; samples were deidentified for patient confidentiality. RNA was isolated from selected samples using High Pure FFPET RNA isolation kit (Roche, Rotkreuz, Switzerland) and further processed in the nCounter FLEX system (NanoString Technologies, Seattle, WA) using our custom circRNA panel[6] (Supplementary Table 1) and following NanoString's guidelines. Differential expression analysis was performed comparing the normalized counts for each circRNA in patients with recurrent versus nonrecurrent NSCLC. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) and further Sanger sequencing were selected as a second method to validate selected differentially expressed circRNAs. To explore the prognostic impact of relevant circRNAs, expression levels between recurrent and nonrecurrent EGFR mutant-positive cases were compared considering recurrence-free survival. ML was performed using random forest (RF) for feature selection. Different classifiers were then tested, and the one having the highest area under the receiver operating characteristic curve value was selected as the final model. The study was carried out in accordance with the principles of the Declaration of Helsinki and its later amendments, under an approved protocol of the Institutional Review Board of the Hiroshima University. Written informed consent was obtained from each participant before their inclusion in the study. For more detailed information, refer to the Supplementary Methods.

Results

A total of 76 patients were identified after molecular confirmation of the EGFR mutation, with 34 of them relapsing within the next three years after resection (Table 1). Relapsing patients tended to be of female sex (n = 21; 61.8%), and most of them reported less smoking history (n = 21; 61.8%). The median age of this cohort was 71.5 (range: 49–89) years. Most cases (n = 17; 50%) presented an exon 21 mutation, whereas 15 (44.1%) and four (11.8%) patients had an exon 19 and exon 18 mutation, respectively.

Table 1. Demographic and Clinical Characteristics of the EGFR Mutation-Positive Early Resected NSCLC Participants of the Study (RFS at 3 y)

Patient Characteristics	Recurrence (n = 34)	Nonrecurrence (n = 42)	Overall (N = 76)
Age, y			
Median	71.5	69.5	70
Range	49-89	48-89	48-89
Male sex, n (%)	13 (38.2)	11 (26.2)	26 (34.2)
Smoking status, n (%)			
Former or current smoker	13 (38.2)	17 (40.5)	30 (39.5)
Never smoker	21 (61.8)	25 (59.5)	46 (60.5)
Surgical procedure, n (%)			
Lobectomy	28 (82.4)	31 (73.8)	59 (77.6)
Segmentectomy	5 (14.7)	9 (21.4)	14 (18.5)
Wedge resection	1 (2.9)	2 (4.8)	3 (3.9)
Predominant histologic feature, n (%)			
Lepidic	2 (5.9)	14 (33.4)	16 (21.0)
Papillary	26 (76.5)	22 (52.4)	48 (63.1)
Acinar	1 (2.9)	3 (7.1)	4 (5.3)
Micropapillary	1 (2.9)	3 (7.1)	4 (5.3)
Solid	4 (11.8)	0 (0.0)	4 (5.3)
Pleural invasion, n (%)			
Positive	17 (50.0)	8 (19.1)	25 (32.9)
Negative	17 (50.0)	34 (80.9)	51 (67.1)
Lymphovascular invasion, n (%)			
Positive	25 (73.5)	9 (21.4)	34 (44.7)
Negative	9 (26.5)	33 (78.6)	42 (55.3)
Intrapulmonary metastasis, n (%)			
Positive	1 (2.9)	0 (0.0)	1 (1.3)
Negative	33 (97.1)	42 (100.0)	75 (98.7)
EGFR mutation[a] n (%)			
Exon18	4 (11.8)	4 (9.5)	8 (10.5)
Exon19	15 (44.1)	13 (38.1)	28 (36.8)
Exon21	17 (50.0)	23 (54.7)	40 (52.7)
Pathologic stage			
IA1-3/IB	13 (38.2)	34 (80.8)	47 (61.8)
IIA/IIB	5 (14.7)	3 (7.4)	8 (10.6)
IIIA/IIIB	16 (47.1)	5 (11.8)	21 (27.6)

RFS, recurrence-free survival.
[a]Nonrecurrence and recurrence cohorts included one case and two cases harboring double somatic mutations, respectively.

Differential expression analysis was performed finding hsa_circ_0002360 (circRUNX1), along with hsa_circRNA_101367 (circFUT8) and hsa_circRNA_406483 (circAASDH), up-regulated in the relapsing cohort ($p < 0.05$ and >2 fold-change). Among them, circRUNX1 was the most consistently expressed across individuals of the relapsing cohort, with a $-\log_{10}$ p value superior to 8 (Fig. 1*A*). Expression of circRUNX1 was also validated both in relapsing and nonrelapsing cohorts by RT-qPCR and further Sanger sequencing of the circRNA junction site (Fig. 1*B*). By using divergent primers (Supplementary Table 2), our results revealed the presence of circRUNX1 in both nonrelapsing and relapsing cohorts. Although a trend on higher ΔΔCts in the relapsing versus nonrelapsing patients was observed, the difference on circRUNX1 expression between cohorts detected by RT-qPCR was not big enough to be considered statistically significant (Mann-Whitney U test, $p = 0.3845$) in comparison to nCounter results (Mann-Whitney U test, $p < 0.0001$), indicating a higher sensitivity of the latter toward the detection of circRUNX1 (Fig. 1*C*).

Examination of circRUNX1, circFUT8, and circAASDH was also performed in 22 patients with EGFR-wild type (EGFR-WT) NSCLC (Supplementary Table 3), finding expression of the three previously cited circRNAs in all samples analyzed. Differential expression analysis was carried out comparing patients with EGFR-WT NSCLC relapsing within 36 months (n = 12) with those free of disease (n = 10). Of the three circRNAs analyzed, circAASDH was found the only one up-regulated in relapsing EGFR-WT patients (Supplementary Table 4).

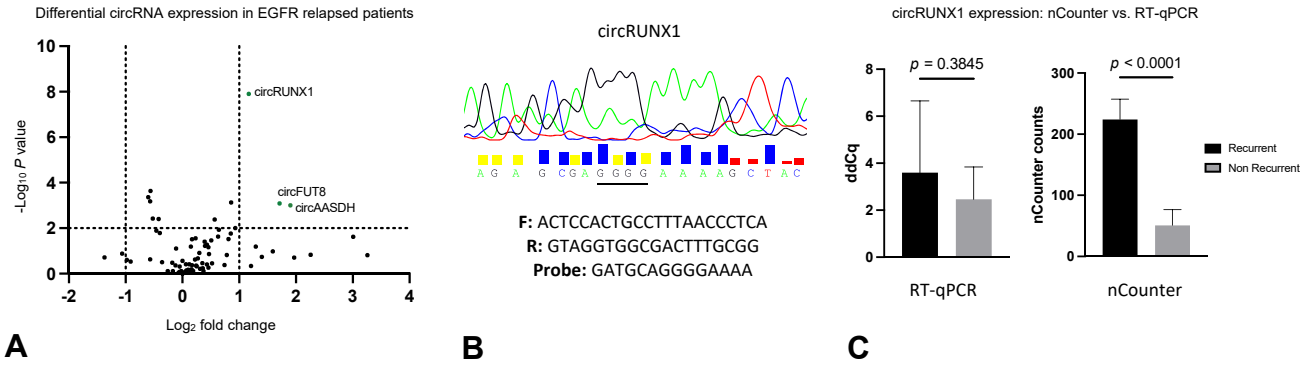

Figure 1. Differential circRNA expression analysis in EGFR mutation-positive early resected NSCLC patients. (*A*) Volcano plot of the circRNA fold-change in relapsing patients versus those patients that did not relapse at 36 months. (*B*) Sanger sequencing results spanning the junction site (underlined) of circRUNX1. (*C*) Bar plot of RT-qPCR versus nCounter results depicting upregulation of circRUNX1 in recurrent EGFR mutation-positive NSCLC patients. Bars indicate the mean of the samples included in each cohort. Error bars indicate standard deviation. RT-qPCR, quantitative reverse transcription polymerase chain reaction.

ML was used for the development of a signature predictive of cancer recurrence on patients with EGFR-mutant NSCLC. RF selected six circRNAs, namely circSOX13, circVRK1, circRUNX1, circFUT8, circEPB41L2, and circLYPLAL1, as the best features to comprise the signature. Different combinations of selected features with ETC, RF, GBM, and KNN algorithms were tested. As a result, combination of selected 6-circRNA signature with RF was chosen as a final model, outperforming the other algorithms with an accuracy of 83%, an area under receiver operating characteristic curve of 0.91, sensitivity of 79%, and specificity of 86% (Fig. 2*A* and Supplementary Table 5).

Relapse-free survival was significantly shorter for the subgroup of patients with high versus low circRUNX1 expression (Fig. 2*B*). In addition, comparison of circRUNX1 expression and the ML-generated signature indicated a similar performance of the latter predicting the relapse of the disease (Fig. 2*C*).

Multivariate analysis identified circRUNX1 expression and ML-generated signature as statistically significant prognostic factors with regard to DFS (circRUNX1 HR = 4.420, confidence interval [CI]: 1.710–6.839, $p < 0.001$; ML signature HR = 3.434, CI: 1.683–7.005, $p < 0.001$) along with the pathologic stage (Supplementary Tables 6 and 7).

Discussion

CircRNAs are covalently closed single-stranded RNA molecules which have been found to regulate gene expression (including regulation of their parental gene) through a wide variety of mechanisms, such as miRNA sponging, among others.[5,6] Previous studies have linked aberrant circRNA expression and oncogenesis, positioning circRNAs as potential prognostic biomarkers of the disease. In the context of NSCLC, recent publications have indicated a correlation of EGFR mutation status with high expression of

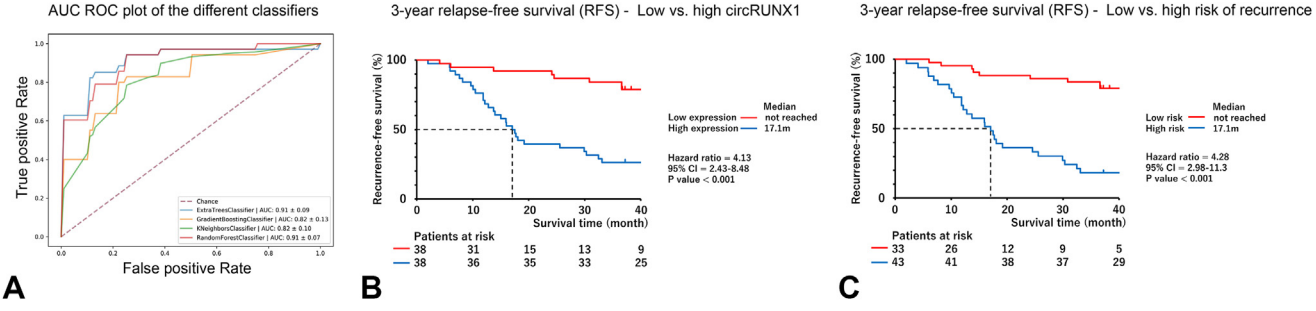

Figure 2. CircRNA analysis in EGFR mutation-positive early-resected NSCLC patients. (*A*) AUC ROC plot of the different classifiers tested with the 6-circRNA signature predictive of NSCLC recurrence in EGFR mutation-positive patients. (*B*) Kaplan-Meier comparing the relapse-free survival of patients showing low versus high expression of circRUNX1. (*C*) Kaplan-Meier comparing the relapse-free survival of patients with low versus high risk of relapse according to the predictive ML circRNA signature. AUC, area under the receiver operating characteristic curve; ROC, receiver operating characteristic.

hsa_circ_0000190 (C190),[7] being the latter a consequence of hyperactivation of EGFR and downstream MAPK signaling.[8]

In the current study, we studied a cohort of 76 Japanese patients with resected EGFR-mutant NSCLC to identify expression patterns that could allow us to predict disease recurrence within 36 months. As a result, circRUNX1 overexpression in these patients was identified as a predictor of lower DFS compared with those with low circRUNX1 expression (HR = 4.13; 95% CI: 2.43–8.48, $p < 0.0001$).

Further ML analysis unveiled a 6-circRNA signature, including circRUNX1, that was highly predictive of DFS (HR = 4.28, 95% CI: 2.98–11.3, $p < 0.001$) (Fig. 2) (Supplementary Tables 6 and 7).

We have previously revealed circRUNX1 overexpressed in lung cancer specimens compared with noncancer controls.[6] Throughout this study, we found that expression of circRUNX1 is superior in relapsing EGFR-mutant versus nonrelapsing patients; however, this pattern was not observed in the EGFR-WT cohort (Supplementary Table 5). These findings further support the prognostic value of circRUNX1 as biomarker of disease recurrence in patients with EGFR-mutant NSCLC.

RUNX1 is a master transcriptional factor interacting with multiple signaling pathways.[9] In metastatic castration-resistant prostate cancer, RUNX1 overexpression, in conjunction with the aryl hydrocarbon receptor, is driven by noncanonical Wnt signaling mediated by ROR1.[10] Interestingly, aryl hydrocarbon receptor and ROR1 overexpression have been associated with resistance to EGFR tyrosine kinase inhibitors and poor PFS, respectively, in patients with NSCLC.[11,12]

A possible mechanism of action has already been described for circRUNX1 regulating the PHF19 through has-miR-3620-5p in lung cancer.[5] In addition, seven miRNAs, namely hsa-miR-1273, hsa-miR-127-5p, hsa-miR-145, hsa-miR-585, hsa-miR-629, hsa-miR-758, and hsa-miR-933, have been predicted by circinteractome (https://circiteractome.nia.nih.gov) to potentially bind to circRUNX1. Further research is warranted to unveil the biological roles of circRUNX1 in EGFR-mutant lung cancer and whether there could be a correlation between the expression levels of both the linear and circular forms of RUNX1.

CircRUNX1 represents a putative novel biomarker in EGFR-mutant NSCLC and could be of interest for those patients undergoing osimertinib adjuvant therapy. We previously investigated circRNA expression from plasma for the detection of early stage NSCLC.[13] Future studies guarantee the assessment of circRUNX1 in plasma of patients with surgically resected EGFR-mutant NSCLC as prognostic biomarker of disease recurrency.

This was a proof-of-concept study. Therefore, the number of samples was quite limited. A prospective study including bigger independent cohorts is foreseen for the training and validation of presented signature.

CRediT Authorship Contribution Statement

Carlos Pedraz-Valdunciel: Conceptualization, Methodology, Writing—Original draft preparation.

Masaoki Ito: Writing—Original draft preparation.

Stavros Giannoukakos: Visualization, Supervision.

Ana Giménez-Capitán: Visualization, Supervision.

Miguel Ángel Molina: Project administration, Supervision.

Rafael Rosell: Visualization, Supervision, Writing—Reviewing and Editing.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *JTO Clinical and Research Reports* at www.jtocrr.org and at 10.1016/j.jtocrr.2023.100604.

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