DDR2 polymorphisms and mRNA expression in lung cancers of Japanese patients

HIDEFUMI SASAKI, MASAYUKI SHITARA, KEISUKE YOKOTA, KATSUHIRO OKUDA, YU HIKOSAKA, SATORU MORIYAMA, MOTOKI YANO and YOSHITAKA FUJII

Department of Oncology, Immunology and Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Received February 3, 2012; Accepted April 13, 2012

DOI: 10.3892/ol.2012.684

Abstract. Discoidin domain receptor 2, DDR2, is a tyrosine kinase receptor for fibrillar collagen that is involved in post-natal development, tissue repair and primary and metastatic cancer progression. Recently, mutations in the *DDR2* kinase gene were identified in squamous cell lung cancer from large-scale Sanger sequencing. The present study investigated the *DDR2* gene mutations and mRNA expression in surgically treated non-small cell lung cancer (NSCLC) of squamous histology cases. The presence or absence of *DDR2* mutations at the kinase and discoidin domain was analyzed by direct sequencing. In this cohort, *DDR2* mutations were not observed in the 166 patients with lung cancer, although *DDR2* polymorphisms were observed (H136H, n=14) at the discoidin domain. mRNA levels of *DDR2* in lung tumor samples and the adjacent normal lung samples were simultaneously analyzed. *DDR2* mRNA levels were significantly decreased in tumor samples compared with normal lung samples. However, the *DDR2* mRNA levels were elevated in the *DDR2* polymorphism cases.

Introduction

Lung cancer is a major cause of mortality from malignant disease, due to its high incidence, malignant behavior and the lack of major advancements in treatment strategy (1). A great deal of progress has been made in the target therapy for non-small cell lung cancer (NSCLC), largely owing to the development of small molecular inhibitors, including epidermal growth factor receptor (EGFR) (2-6) and ALK (7). By contrast, patients with the principal subtype of NSCLC, squamous cell lung cancer, rarely respond to these agents. Mutations in the discoidin domain receptor (DDR) kinase gene have been identified in squamous cell lung cancer from large-scale Sanger sequencing (8). DDRs have been shown to exhibit altered expression patterns in multiple types of human cancer, including lung cancers (9,10). The mechanism by which the receptors may contribute to oncogenesis is not well known; however, given the important role of the receptors in transmitting signals from the extracellular matrix (ECM), it is possible that the receptors act as regulators of cell proliferation, adhesion, migration and tumor metastasis (9). The downregulation of *DDR2* mRNA expression has been reported in NSCLCs (10). Davies *et al* screened for mutations in patients with lung cancer by comprehensively sequencing 518 kinases in the human genome and described a novel *DDR2* gene mutation (R105S) in the discoidin domain (11). However, the mutation status of the *DDR2* gene in the Japanese population has not been well reported.

In this study, the mutation status of *DDR2* at the discoidin and kinase domains in lung squamous histology tumors was investigated. This study also investigated *DDR2* mRNA levels via real-time PCR using LightCycler. The findings were compared with the clinicopathological features of lung cancer.

Patients and methods

Patients. The study group included patients with lung cancer who had undergone surgery at the Department of Surgery II, Nagoya City University Hospital (Nagoya, Japan). Patient consent was obtained from the patients or a family member. The study wa approved by the ethics committee of the hospital. Tumor samples were immediately frozen and stored at -80˚C until they were assayed. Hammerman *et al* demonstrated that the *DDR2* mutations were present within the squamous histology of lung cancer (8), therefore this study focused on squamous cell carcinomas. The clinical and pathological characteristics of the 166 patients with lung cancer for *DDR2* gene analyses were as follows: 143 (86.1%) were male and 23 were female; 143 (86.1%) were diagnosed with squamous cell carcinomas and 22 were adenosquamous cell carcinomas; 153 (92.2%) were smokers and 13 were non-smokers. The clinical and pathological characteristics of the 92 patients with lung cancer for *DDR2* gene mRNA expression analyses were as follows: 80 (87%) were male and 12 were female; 87 (94.6%) were diagnosed with squamous cell carcinomas

Correspondence to: Dr Hidefumi Sasaki, Department of Oncology Immunology and Surgery, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
E-mail: hisasaki@med.nagoya-cu.ac.jp

Key words: discoidin domain receptor 2, mRNA expression, lung cancer, squamous cell carcinoma, polymorphism

and four were adenosquamous cell carcinomas; five (5.4%) were light-smokers (Brinkman index <100) and 52 (56.5%) were of pathological stage I.

PCR assays for DDR2 mRNA expression. Total RNA was extracted from lung cancer tissues using an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The RNA concentration was determined using a spectrophotometer and adjusted to a concentration of 200 ng/ml. Approximately 10 cases were excluded for each assay as there were not enough tumor cells to extract sufficient tumor RNA. RNA (1 μg) was reverse transcribed using Superscript II enzyme (Gibco-BRL, Gaithersburg, MD, USA) with 0.5 μg oligo $(dT)_{12\text{-}16}$ (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The reaction mixture was incubated at 42˚C for 50 min and then at 72˚C for 15 min. PCR analyses were performed using 1 μl of each DNA sample. The PCR was performed using LA-Taq kit (Takara Bio Inc., Shiga, Japan) in a 25-μl reaction vessel. The primer sequences for the discoidin domain of the *DDR2* gene were as follows: forward: 5'-CAGCTTCCAGTCAGTGGTCA-3' and reverse: 5'-GCCAGCCCACATAGTCATAG-3' (643 bp, exons 3-7). The cycling conditions were as follows: initial denaturation at 94˚C for 5 min, followed by 40 cycles at 94˚C for 45 sec, 60˚C for 45 sec and 72˚C for 45 sec. The primer sequences for the *DDR2* gene kinase domain were as follows: forward: 5'-TTTGGGGAGGTTCATCTCTG-3' and reverse: 5'-GTCAGGACAAATGGCTGGTT-3' (747 bp, exons 9-12). The cycling conditions were as follows: initial denaturation at 94˚C for 5 min, followed by 40 cycles at 94˚C for 45 sec, 60˚C for 45 sec and 72˚C for 45 sec. The products were purified by a Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). The samples were sequenced using the ABI prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms via manual review.

To ensure the fidelity of mRNA extraction and reverse transcription, the samples were subjected to PCR amplification with oligonucleotide primers specific for the constitutively expressed gene β-actin and normalized using a β-actin detection kit (Nihon Gene Research Laboratories, Miyagi, Japan). The primer sequences for the *DDR2* gene were as follows: forward: 5'-CCACTATGCAGAGGCTGACA-3' and reverse: 5'-CAGAGATGAACCTCCCCAAA-3' to amplify a 183-bp fragment. The cycling conditions were as follows: initial denaturation at 95˚C for 10 min, followed by 50 cycles at 95˚C for 5 sec, 60˚C for 5 sec and 72˚C for 8 sec. PCR reactions were performed using a LightCycler-FastStart DNA Master SYBR-Green I kit (Roche Molecular Biochemicals, Mannheim, Germany) and quantified.

Statistical analysis. Statistical analysis was performed using the Mann-Whitney U test for unpaired samples and Wilcoxon's signed-rank test for paired samples. Linear correlations between the variables were determined by means of a simple linear regression. Correlation coefficients were determined by rank correlation using the Spearman's test and χ^2 test. The overall survival rate of patients with lung cancer was examined using the Kaplan-Meier method and differences were examined using the log-rank test. Analyses were performed using the Stat-View software package (Abacus Concepts Inc., Berkeley, CA, USA). P<0.05 was considered to indicate a statistically significant result.

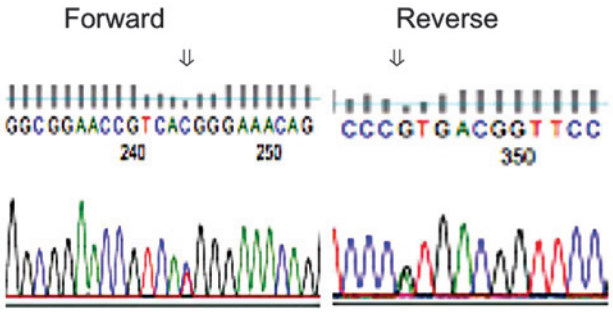

Figure 1. *DDR2* polymorphism at the discoidin domain, histidine136 to histidine, at nucleotide 408 (T to C change). Left panel, forward sequence from lung cancer samples. Right panel, reverse sequence. DDR2, discoidin domain receptor 2.

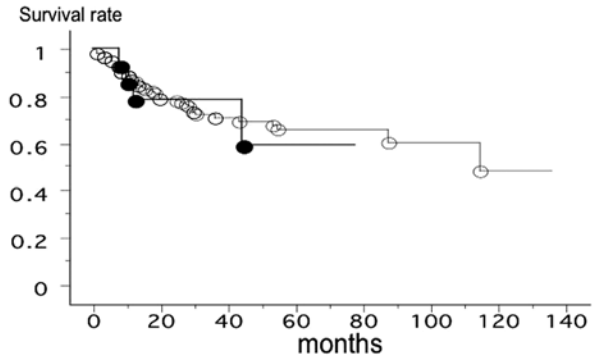

Figure 2. The overall survival rate of the 162 patients with lung cancer from Nagoya City University, with follow-up until August 31, 2011, was studied with reference to the *DDR2* gene status. The survival of patients with the *DDR2* gene polymorphism (n=14, 5 mortalities) and patients with the wild-type *DDR2* (n=148, 41 mortalities) was not significantly different (log-rank test, p=0.7944). DDR2, discoidin domain receptor 2. Open circle, wild-type cases; closed circle, polymorphism cases.

Results

DDR2 gene mutation status in Japanese patients with lung cancer. The current study sequenced the kinase domain of *DDR2* gene for 173 squamous histology NSCLC samples. Of 173 patients, from direct sequencing using cDNA samples, no mutations were identified. This study also sequenced the discoidin domain of the *DDR2* gene for 166 squamous histology NSCLC samples, where 148 samples overlapped. Of 166 patients, from direct sequencing using cDNA samples, no mutations were identified. However, 14 *DDR2* polymorphism cases (8.4%) were identified (Fig. 1). The nucleotide T at 408 was changed to C. The amino acid was not converted from histidine (CAT>CAC, His>His). This *DDR2* polymorphism status was not correlated with gender (male 11/143 vs. female; 3/23, p=0.3914), age (age ≤65, 7/71 vs. >65, 7/104; p=0.5724) or smoking status (smoker 14/153 vs. non-smoker 0/13; p=0.2544). The polymorphism cases tended to be higher in squamous cell carcinomas (p=0.0796) when compared with the adenosquamous carcinomas. The polymorphism cases

Table I. Clinicopathological data of 92 patients with lung cancer.

		DDR2	
Factors	No. of patients n (%)	T-N ratio of DDR2/β-actin mRNA levels	P-value
Mean age (years)	66.7±9.0	92	
Stage			
I	52 (56.5)	0.978±0.187	NS
II	21 (22.8)	1.027±0.369	
III	18 (19.6)	1.532±0.611	
IV	1 (1.1)	0.17	
Tumor status			
T1	30 (32.6)	0.884±0.240	NS
T2	34 (37.0)	0.990±0.232	
T3	8 (8.7)	1.295±0.406	
T4	10 (10.8)	1.975±1.048	
Lymph node metastasis			
N0	65 (70.7)	1.025±0.162	NS
N1	15 (16.3)	1.682±0.835	
N2	12 (13.0)	0.753±0.272	
Pathological subtype			
Adenosquamous	5 (5.4)	0.676±0.423	0.5839
SCC	87 (94.6)	1.113±1.765	
DDR2 polymorphism			
T408C	7 (8.2)	3.301±3.972	0.0465
T408T	78 (91.8)	0.929±1.717	
Smoking			
BI<100	5 (5.4)	0.386±0.368	0.1479
BI>100	78 (94.6)	1.129±1.760	
Age (years)			
≤65	35 (38.0)	1.318±1.716	0.3204
>65	57 (62.0)	0.948±1.724	
Gender			
Male	80 (87.0)	1.115±1.823	0.3272
Female	12 (13.0)	0.916±0.765	

T-N, tumor-normal; NS, not significant; SCC, squamous cell carcinoma; BI, Brinkman index; DDR2, discoidin domain receptor 2.

were predominantly observed in advanced stages (stage II-IV, 11/74 vs. stage I, 3/92; p=0.0075).

DDR2 gene expression status in Japanese patients with lung cancer. In the 92 tissues from histologically confirmed lung squamous cell carcinoma, the mean value for *DDR2* mRNA level as standardized by the mRNA level of β-actin (10.915±1.546, mean ± standard deviation) was significantly lower than the tissues from non-malignant lung tissue (22.790±3.382, p=0.0013). The T/N ratios of the *DDR2* mRNA levels for each sample were: stage I, 0.978±0.187; stage II, 1.027±0.369; stage III, 1.532±0.611; and stage IV, 0.17 (Table I). The *DDR2* mRNA T/N ratios in squamous cell carcinoma (1.113±1.765) and adenosquamous cell carcinomas (0.676±0.423) were not significantly different (p=0.5839). No significant difference in *DDR2* mRNA levels T/N ratio was observed between gender and age. The patient groups were further stratified according to clinicopathological factors. The T/N ratio was not significantly different between the light-smokers (Brinkman index <100; 0.386±0.368) and smokers (>100; 1.129±1.765; p=0.1479). The T/N ratio of *DDR2* mRNA levels in each sample were: T1, 0.884±0.240; T2, 0.990±0.232; T3, 1.295±0.406; and T4, 1.975±1.048. Although there was no significant difference, *DDR2* mRNA levels were higher in advanced T stages (Table I).

Correlation between DDR2 and DDR2 polymorphisms. Of the 85 patients that overlapped, 7 polymorphism cases were found at the discoidin domain (C408T, H136H; Fig. 1). The *DDR2* polymorphism in lung cancer had a significantly higher *DDR2* mRNA level (T/N ratio, 3.301±3.972) than the wild-type *DDR2* for lung cancer (T/N ratio, 0.929±1.717; p=0.0465).

The overall survival rate of 162 patients with lung cancer from Nagoya City University, with follow-up until August 31, 2011, was studied with reference to the *DDR2* gene status. The survival rate of patients with the *DDR2* gene polymorphism (n=14, 5 mortalities) and the patients with the wild-type *DDR2* (n=148, 41 mortalities) was not significantly different (log-rank test, p=0.7944; Fig. 2).

Discussion

The present study has shown that *DDR2* mRNA expression is significantly deregulated in NSCLC when compared with normal lung tissue. However, *DDR2* mRNA levels were higher in the *DDR2* polymorphism cases. The polymorphism cases (8.4%) were predominantly observed within the advanced lung cancers. The collagen-binding RTK DDRs have previously been linked to various human diseases, including cancers (12-15). Although the sample size was not large, no *DDR2* mutations were observed in this cohort. However, the *DDR2* expression pattern in lung cancer suggests that *DDR2* contributes to the pathogenesis of lung cancer.

Previous studies have reported somatic mutations in the *DDR2* gene at the discoidin or kinase domain (8,11), however, the present study did not confirm the existence of the *DDR2* mutation. There are several explanations for this discrepancy. Lung cancer encompasses a broad range of clinical subtypes, in which the two cohorts differed. In addition, an ethnic difference between the studies on mutant *DDR2* may exist, as in *EGFR* gene mutations (2-6). The present study cannot conclude that differences in the *DDR2* sequence were due to methodology or PCR and sequencing methods. However, this study detected 8.4% of polymorphism cases at the discoidin domain in this cohort.

The mechanism by which DDRs may contribute to oncogenesis is not well known, however, given their role in transmitting signals from the ECM, it is likely that DDRs act as regulators of cell proliferation, adhesion, migration and subsequent tumor metastasis. Prolonged stimulation of DDR2 is associated with the upregulation of MMP-1 expression (16). DDR2 is also important in mediating fibroblast migration and proliferation via a MMP-2-dependent mechanism (17,18). Activated DDR2 has been noted to induce the expression of MMP-1, MMP-2 and MMP-13 (17,19). Similar to EGFR, it is conceivable that an altered expression of DDRs triggers abnormal activity, ultimately leading to enhanced proliferation and oncogenesis. In this study, the synonymous nucleotide change in *DDR2* was present in approximately 10% of the present clinical cohort. This polymorphism was correlated with an increased *DDR2* expression and advanced pathological stages of lung cancers.

It has been reported that the development of experimental liver metastasis using melanoma cells, which were stably transfected with a small interfering RNA for *DDR2*, was reduced compared with mock transfected clones (20). Findings of a previous study revealed that imatinib, nilotinib and dasatinib are potent inhibitors of the kinase activity of DDRs (21). The kinase profiles of the three compounds were addressed in two chemical proteomic studies which observed that the compounds also bind to the DDRs (21,22). Of these compounds, dasatinib was the most potent inhibitor of DDRs (17). Recently, it was observed that only dasatinib produced a response in mutant-*DDR2* lung cancer cell lines (8). These findings may contribute to a greater understanding of the therapeutic potential of these inhibitor compounds with DDR2.

In conclusion, the *DDR2* mutation in lung cancers of Japanese patients was observed to be extremely rare. However, the *DDR2* polymorphism and/or its expression may be involved in the progression of lung cancer.

Acknowledgements

The authors thank Mrs. Miki Mochizuki for her technical assistance. This study was supported by Grants-in-Aid for Scientific Research, Japan Society for the Promotion of Science (JSPS; Nos. 23659674, 24592097, 21591820) and a grant for the Cancer Research Program for Developing the Supporting System for Upgrading Education and Research (2009) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Ginsberg RJ, Kris K and Armstrong G (eds): Cancer of the lung. In: Principles and Practice of Oncology. 4th edition. Lippincott, Philadelphia, pp673-682, 1993.
2. Paez JG, Janne PA, Lee JC, *et al*: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304: 1497-1500, 2004.
3. Lynch TJ, Bell DW, Sordella R, *et al*: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350: 2129-2139, 2004.
4. Sasaki H, Endo K, Konishi A, *et al*: EGFR mutation status in Japanese lung cancer patients: genotyping analysis using LightCycler. Clin Cancer Res 11: 2924-2929, 2005.
5. Sasaki H, Shimizu S, Endo K, *et al*: EGFR and erbB2 mutation status in Japanese lung cancer patients. Int J Cancer 118: 180-184, 2006.
6. Fukuoka M, Wy YL, Thongprasert S, *et al*: Biomarker analyses and first overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). J Clin Oncol 29: 2866-2874, 2011.
7. Soda M, Choi YL, Enomoto M, *et al*: Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 448: 561-566, 2007.
8. Hammerman PS, Sos ML, Ramos AH, *et al*: Mutations in the DDR2 kinase gene identify a novel therapeutic target in squamous cell lung cancer. Cancer Discovery 1: 78-89, 2011.
9. Vogel WF, Abdulhussein R and Ford CE: Sensing extracellular matrix: an update on discoidin domain receptor function. Cell Signal 18: 1108-1116, 2006.
10. Ford CE, Lau SK, Zhu CQ, *et al*: Expression and mutation analysis of the discoidin domain receptors 1 and 2 in non-small cell lung carcinoma. Br J Cancer 96: 808-814, 2007.
11. Davies H, Hunter C, Smith R, *et al*: Somatic mutations of the protein kinase gene family in human lung cancer. Cancer Res 65: 7591-7595, 2005.
12. Johnson JD, Edman JC and Rutter WJ: A receptor tyrosine kinase found in breast carcinoma cell has an extracellular discoidin I-like domain. Proc Natl Acad Sci USA 90: 5677-5681, 1993.
13. Alves F, Vogel W, Mossie K, *et al*: Distinct structural characteristics of discoidin I subfamily receptor tyrosine kinases and complementary expression in human cancer. Oncogene 10: 609-618, 1995.
14. Barker KT, Martindale JE, Mitchell PJ, *et al*: Expression patterns of the novel receptor-like tyrosine kinase, DDR, in human breast tumors. Oncogene 10: 569-575, 1995.
15. Nemoto T, Ohashi K, Akashi T, *et al*: Overexpression of protein tyrosine kinases in human esophageal cancer. Pathobiology 65: 195-203, 1997.

16. Vogel W, Gish GD, Alves F, *et al*: The discoidin domain receptor tyrosine kinases are activated by collagen. Mol Cell 1: 13-23, 1997.
17. Olaso E, Labrador JP, Wang L, *et al*: Discoidin domain receptor 2 regulates fibroblast proliferation and migration through the extracellular matrix in association with transcriptional activation of matrix metalloproteinase-2. J Biol Chem 277: 3606-3613, 2002.
18. Olaso E, Ikeda K, Eng FJ, *et al*: DDR2 receptor promotes MMP-2-mediated proliferation and invasion by hepatic stellate cells. J Clin Invest 108: 1369-1378, 2001.
19. Xu L, Peng H, Wu D, *et al*: Activation of the discoidin domain receptor 2 induces expression of matrix metalloproteinase 13 associated with osteoarthritis in mice. J Biol Chem 280: 548-555, 2005.
20. Badiola I, Villacé P, Basaldua I, *et al*: Downregulation of discoidin domain receptor 2 in A375 human melanoma cells reduces its experimental liver metastasis ability. Oncol Rep 26: 971-978, 2011.
21. Day E, Waters B, Spiegel K, *et al*: Inhibition of collagen-induced discoidin domain receptor 1 and 2 activation by imatinib, nilotinib and dasatinib. Eur J Pharmacol 599: 44-53, 2008.
22. Bantscheff M, Eberhard D, Abraham Y, *et al*: Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. Nat Biotech 25: 1035-1044, 2007.