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Functional analysis of *Discoidin domain receptor 2* mutation and expression in squamous cell lung cancer

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ABSTRACT

Objectives: Discoidin domain receptor (DDR) 2 mutations have recently been reported to be candidate targets of molecular therapy in lung squamous cell carcinoma (SQCC). However, the status of DDR2 expression and mutations, as well as their precise roles in lung SQCC, have not been clarified. We here report *DDR2* mutation and expression status in clinical samples and its role of lung SQCC.

Materials and methods: We investigated *DDR2* expression and mutation status in 44 human clinical samples and 7 cell lines. Biological functions of DDR2 were assessed by *in vitro* cell invasion assay and animal model experiments.

Results: Endogenous DDR2 protein expression levels were high in one cell line, PC-1, and immunohistochemistry of lung cancer tissue array showed high levels of DDR2 protein in 29% of lung SQCC patients. A mutation (T681I) identified in lung SQCC and the cell line EBC-1 was detected among 44 primary lung SQCC samples and 7 lung SQCC cell lines. Although Forced expression of DDR2 and its mutant (T681I) led to induce SQCC cell invasion *in vitro,* only wild type DDR2 enhanced lung metastasis in an animal model. We also found that ectopic expression of *DDR2* induced MMP-1 mRNA expression accompanied by phosphorylation of c-Jun after treatment with its ligand, collagen type I, but DDR2 with the T681I mutation did not, suggesting that T681I mutation is an inactivating mutation.

Conclusion: Overexpression of *DDR2* might contribute to tumor progression in lung SQCC. The overexpression of DDR2 could be potential molecular target of lung SQCC.

1. Introduction

Lung cancer treatment strategies, especially molecular targeted therapy, have progressed rapidly. However, most established treatments are for adenocarcinoma of the lung, not for squamous cell carcinoma (SQCC). SQCC constitutes approximately 30% of lung cancers, with a prognosis that is worse than that of adenocarcinoma [1,2]. The number of molecular alternations in SQCC is larger than in adenocarcinoma, presumably because SQCC comprises a larger proportion of smokers [3]. A large comprehensive cohort study of lung SQCC patients was conducted as part of The Cancer Genome Atlas (TCGA) project [4]; in that study, the mean somatic mutation rate was 8.1 per megabase (Mb), higher than with acute myelogenous leukemia (0.56 per Mb), breast carcinoma (1.0 per Mb), and colorectal carcinoma (3.2 per Mb). Considering these findings, difficulty treating SQCC is thought to be due mainly to various genetic alterations, so that it is unlikely that driver mutations exist. Based on that, discoidin domain receptor (DDR) 2 mutations and FGFR1 amplification have recently been reported from single-platform studies as candidate targets for molecular therapy [5,6].

The *DDR2* gene is located in 1q23.3, and its product is a tyrosine kinase receptor for fibrillar collagen [7–10]. Under normal physiological conditions, it acts in cutaneous wound healing mediated by proliferation, chemotactic migration, and secretion of metalloproteinases and collagen by skin fibroblasts, and it also acts in bone development [11]. DDRs have also been implicated in cancer progression, and *DDR2* mutations have been found in SQCCs [5]. Some of these mutations promote colony formation by NIH-3T3 cells in soft agar with the ectopic expression system, and shRNA targeting DDR2 reduces proliferation in

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cell lines carrying *DDR2* mutation, suggesting that some *DDR2* mutations, such as L63 V in the discoidin domain and I638F in the tyrosine kinase domain, have oncogenic properties [5]. DDR2 mutations are found in 4–5% of SQCCs and are located in at least fifteen different regions including the discoidin domain and the tyrosine kinase domain [5,12,13]. Although Dasatinib, second generated BCR-Abl inhibitor, has already been actually used in clinical practice for chronic myeloid leukemia, it also inhibits tumor growth of lung SQCC cell lines that have *DDR2* mutations [5,10]. Based on these results, it is concluded that DDR2 is a candidate for molecular targeting in lung SQCCs. However, *DDR2* expression and mutation status, as well as the precise role of *DDR2* in lung SQCC, have yet to be clarified.

In this paper, we describe our investigation of *DDR2* mutation status and DDR2 protein level in patients with lung SQCC and our examination of the biological function of DDR2 *in vitro* and in animal model experiments. DDR2 was overexpressed in 29% of lung squamous cell carcinoma, and mutations were sporadically observed. DDR2 overexpression promoted SQCC cell invasion *in vitro* and metastasis in the animal model, and those activities were reduced with the T681I *DDR2* mutation, which we found among 44 primary lung SQCC samples and 7 lung SQCC cell lines. DDR2 phosphorylation induced by collagen treatment increased gene expression of several genes, including *MMP-1*, and c-Jun phosphorylation. We discuss the implications of these results for the possible use of DDR2 as a molecular target in the treatment of lung SQCC.

2. Material and methods

2.1. Tissue samples and lung cancer cell lines

Tissue samples for analysis of DDR2 mutation status were obtained from surgical or biopsy specimens of 44 SQCC patients who underwent treatment at Saga University Hospital between 2004 and 2012. The study protocol was approved by the Clinical Research Ethics Committee of Saga University and performed in accordance with the guidelines and regulations. All patients gave informed consent for obtaining tissue specimens according to the Declaration of Helsinki.

Human squamous cell lung cancer cell line H226 B was provided by Dr. Jonathan Kurie at University of Texas M.D. Anderson Cancer Center, Houston, TX, USA. Calu-1 was purchased from American Type Culture Collection (Manassas, VA). LK2, RERF-LC-AI, QG-56, PC-1, and EBC-1 were provided by Dr. Tomonori Hayashi of the Radiation Effects Research Foundation, Hiroshima, Japan and, Cells were cultured in RPMI1640 containing 10% fetal bovine serum (FBS).

2.2. DNA extraction and DDR2 sequencing analysis

Genomic DNA was isolated by using the QIAamp® DNA mini kit (QIAGEN, Hilden, Germany). *DDR2* from exons 1–18 was sequenced by PCR-based direct sequencing. PCR amplification was performed with Discoverase™ DHPLC DNA polymerase (Invitrogen Inc., CA). The amplified product was sequenced directly using the Applied Biosystems PRISM dye terminator cycle sequencing method with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.3. Antibodies for immunostaining and western blotting

The following antibodies were used: DDR2 (R & D Systems, Minneapolis, MN, USA, AF2538 and Cell Signaling(CS) #12133), Actin (CS #4970 and Santa Cruz sc-1616), FLAG (Sigma-Aldrich, St Louis, MO, USA, F3165), Phosphotyrosine (Millipore 05-321), Phospho-c-Jun Ser63 (CS #9261), Phospho-c-Jun Ser73 (CS #9164), c-Jun (Santa Cruz sc-1694), Phospho-JNK Thr183/Tyr185 (CS #9251), JNK (CS #9252), Phospho-p38 MAPK Thr180/Tyr182 (CS #9211), and p38α MAPK (Santa Cruz sc-535).

2.4. Western blotting analysis

Whole cell lysates were prepared from cell lines using lysis buffer containing Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate, and 1% sodium deoxycholate with both phosphatase and protease inhibitors. The cell lysates were subjected to NuPAGE and transferred onto a nitrocellulose membrane (Schleicher & Schell, Inc., Keene, NH). Subsequently, the membranes were blocked, washed, and incubated with primary and secondary antibody. An ECL kit (Amersham Corp., Arlington Heights, IL) was used for detection.

2.5. Immunohistochemistry

We used a tissue array of 195 lung cancers including specimens from 28 patients with SQCC who underwent surgery at Saga University Hospital. Values of immunoreaction intensity were used to quantify cytoplasmic DDR2 expression (0 no staining, 1+ weak, 2+ moderate, 3+ intense labeling) and percentage of tumor cell staining (0–100%). We multiplied immunoreaction intensity by percentage of staining; we defined high DDR2 as greater than or equal to the median (≧182) and low DDR2 as less than the median (< 182).

2.6. Construction of DDR2 and its mutant, T681I expression plasmids

The myc-DDK-tagged ORF clone of Homo sapiens *DDR2* and empty vector were obtained from Origene (Rockville, MD). T681I mutation was introduced by using the QuikChange Site-Directed Mutagenesis Kit and specific oligonucleotides 5′-CCTCCAGCGATGTACGCATTGTCA GTTACACC-3′ (sense), and was verified by direct sequencing. Each plasmid DNA was transfected into H226 B cells by Lipofectamine. After 48 h in culture, G418 (Sigma-Aldrich Co., Tokyo) was added to the medium, and stable transformants with DDR2 (H226B-WT), T681I (H226 B -T681I), or vector alone (H226B-emp) were established.

2.7. Analysis of cell proliferation

After 5×10^2 cells were cultured in each well of a 96-well plate, the number of cells was measured using a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technology, Japan).

2.8. in vitro cell invasion assay

The *in vitro* cell invasion assay with a Matrigel-coated membrane and migration assay without Matrigel membrane were performed using BD BioCoat® Matrigel invasion chambers with 8 μm pore size (BD Biosciences). Cell invasion and migration were induced by FBS, and the numbers of invaded cells or migrated cells were determined by microscopic examination at a 100× magnification for invasion assays and at a 200× magnification for migration assays on any three fields of each membrane.

2.9. Animal experiment

NOD/SCID/JAK3^{null} (NOJ) mouse lines were established as described previously [14,15]. We injected into the dorsal flanks of 8-week-old male NOJ mice 0.5×10^5 cells of H226B-emp, H226B-WT, or H226B-T681I. Tumor volumes and body weight were measured every 3 days. Mice were evaluated daily for signs of morbidity or tumor growth. When body weight decreased by 10% of original weight, the mice were euthanized with ether and dissected. Lungs were fixed in 10% formaldehyde and processed for pathological examination. The aggregate area of lung metastasis (% of total lung) was quantified with the ImageJ software.