Overexpression of CD 133 and BCL-2 in non-small cell lung cancer with neuroendocrine differentiation after transformation in *ALK* rearrangement-positive adenocarcinoma

Running title: Transformation of *ALK*-rearranged NSCLC

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Abstract

Transformation to small cell lung cancer is one phenomenon of acquired resistance to anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors in *ALK* rearrangement-positive non-small cell lung cancer (NSCLC). Few case reports have focused on other types of histological transformation. We report a case of transformation of *ALK* rearrangement-positive adenocarcinoma to NSCLC with neuroendocrine differentiation during alectinib therapy. A 36-year-old woman presented with a tumor in the left lower lobe and bone metastases. She was diagnosed with *ALK* rearrangement-positive adenocarcinoma by histopathology of the primary tumor. Alectinib had been effective for 8 months before new lesions appeared. Histopathological re-examination of a recurrent tumor revealed poorly differentiated carcinoma with insulinoma-associated protein 1 (INSM1) expression, which remained ALK-positive. Expression of CD133, BCL-2, and SOX2 was positive in comparison to the initial tumor. Expression of SOX2 became more strongly positive than it was before treatment. The immunohistochemical findings of these markers associated with cancer stem-like cells and/or neuroendocrine differentiation suggest that cancer stem cells play a role in the mechanisms of histological transformation and acquired resistance of *ALK* rearrangement-positive cancer. To our knowledge, this is the first report to suggest an

association between cancer stem-like cells and histological transformation in *ALK* rearrangement-positive lung cancer.

Keywords: adenocarcinoma, *ALK* rearrangement-positive lung cancer, cancer stem cell, neuroendocrine carcinoma, small cell lung cancer, transformation

Introduction

Anaplastic lymphoma kinase (*ALK*) rearrangement-positive lung cancer accounts for 2% to 7% of all non-small cell lung cancers (NSCLC).[1] The ALK tyrosine kinase inhibitors (ALK-TKI), such as crizotinib and alectinib have high treatment efficiency in patients with *ALK* rearrangement-positive NSCLC.[2] However, patients treated with ALK-TKI eventually develop resistance to these drugs. Several case reports have shown histological transformation to small cell lung cancer (SCLC), which is one of the phenomena associated with the development of resistance;[3] however, the mechanism by which cancer acquires neuroendocrine features remains fully unknown. We report a case of a patient with transformation of *ALK* rearrangement-positive adenocarcinoma to NSCLC with neuroendocrine differentiation. In addition, we investigated the associated pathological features before and after transformation. In this case we focus on the cancer stemness as the mechanisms of TKI resistance, because in our previous study we demonstrated the drug tolerant clones were consisted of cancer stem cells and senescent cells.[4] Although Giroux-Leprieur E et al. reported about the association of cancer stemness and drug resistance in terms of Hedgehog Signaling,[5] there are few previous reports about the correlation of cancer stemness and histological transformation.

Clinical summary

A 36-year-old female never-smoker presented to our hospital because of low back pain. She had no history of serious illness. Physical examination and vital signs were normal. Laboratory data showed elevated levels of carcinoembryonic antigen (CEA) (69.4 ng/mL; reference range, < 5 ng/mL). A chest computed tomography (CT) scan revealed a tumor with diameter of 31 mm in the left lower lobe (Fig. 1a), enlarged left hilar and mediastinal lymph nodes, and a metastatic lesion in the sixth cervical vertebra. A positron emission tomography (PET)-CT scan showed increased fluorodeoxyglucose uptake in these lesions and multiple metastatic bone lesions. A transbronchial lung biopsy of the tumor was performed and histopathological examination revealed adenocarcinoma. The break-apart fluorescent in-situ hybridization (FISH) of the specimen showed rearrangement of the *ALK* gene. The patient was diagnosed with *ALK* rearrangement-positive adenocarcinoma (Stage IVB [cT2aN2M1c]). Targeted therapy with alectinib (600 mg/day) was administered. The primary and metastatic lesions decreased in size (Fig. 1b) and the level of CEA was reduced (1.1 ng/mL) after 3 months of treatment. However, 8 months later, a chest CT scan showed newly enlarged mediastinal lymph nodes, which were different from the enlarged lymph nodes observed at the first diagnosis (Fig. 1c). Magnetic resonance imaging (MRI) of the brain revealed

multiple brain metastases. The level of CEA tended to elevate (6.7 ng/mL), and that of Pro-GRP was high (229.1 pg/mL, reference range, < 81 ng/mL). Transbronchial needle aspiration (TBNA) of the enlarged subcarinal lymph node was performed. Histopathological examination revealed poorly differentiated carcinoma, and immunohistochemical analysis showed positive results for neuroendocrine markers. Histological morphology was different from that of the primary tumor. The patient was then diagnosed with developing histological transformation to NSCLC, favor adenocarcinoma with neuroendocrine differentiation. Second-line chemotherapy with cisplatin–irinotecan combination therapy was administered. After two cycles, the level of Pro-GRP was reduced (57.5 pg/mL). A chest CT scan and brain MRI performed after four cycles of chemotherapy showed size reduction of the mediastinal lymph nodes and brain metastases (Fig. 1d).

Pathological findings

A specimen of the primary tumor obtained by transbronchial lung biopsy before treatment with alectinib showed well- to moderately-differentiated adenocarcinoma, with papillary structure or glandular formation (Fig. 2a). Immunohistochemical analysis showed positive results for thyroid transcription factor (TTF-1) (clone 8G7G3/1) (Fig. 2b), ALK (5A4) (Fig. 2c), and negative results for insulinoma-associated protein 1 (INSM1) (A-8) (Fig. 2d), synaptophysin (MRQ-40), and chromogranin A (DAK-A3). The FISH testing was performed using the Vysis ALK Break-Apart FISH Probe kit (Abbott Molecular Inc., USA), which hybridized the 3′ (red, 300 kb) and 5′ (green, 442 kb) regions of ALK and showed rearrangement of the *ALK* gene.

A specimen of the enlarged subcarinal lymph node obtained by TBNA after recurrence revealed poorly differentiated carcinoma, with polygonal tumor cells and abundant cytoplasm composed of solid sheets or nests without papillary or acinar growth, features that were clearly different from those observed in SCLC (Fig. 2e). The proliferative index evaluated with Ki-67 was 20-40%, the mitotic count was 6 mitoses per 2 mm^2 and no necrosis was observed. Immunohistochemical analysis showed positive results for TTF-1 (weakly positive) (Fig. 2f), INSM1 (Fig. 2h), synaptophysin, and chromogranin A. Expression of BCL-2 (124) and the glycoprotein prominin-1

(CD133) (AC133) became positive, even though they were negative before treatment with alectinib (Fig. 3a, b, d, e). Expression of the sex determining region Y-box 2 (SOX2) (SP56) became more strongly positive than it was before treatment with alectinib (Fig. 3c, f). Expression of p53 (DO-7) was heterogeneously positive, and that of the retinoblastoma protein (Rb) (1F8) was positive before and after treatment with alectinib (Fig. 4a–d). Immunohistochemical staining for ALK was positive (Fig. 2g) and FISH showed rearrangement of the *ALK* gene (Fig. 5).

Discussion

We reported a case of *ALK* rearrangement-positive adenocarcinoma, which transformed to NSCLC, favor adenocarcinoma with neuroendocrine differentiation during alectinib treatment. To our best knowledge, there are no previous reports that focus on cancer stemness in the transformation of ALK-TKI resistant NSCLC.

Several case reports have shown histological transformation in *ALK* rearrangement-positive lung cancer, and in almost all of these cases, the histological features after transformation are those of SCLC.[6-8] To our knowledge, only two case reports have been focused on other types of histological transformation; one was NSCLC with neuroendocrine morphology, and the other was pleomorphic carcinoma.[9, 10] The morphology of our case seems to be different from previous reports in terms of the positivity rate of Ki-67 and favor adenocarcinoma feature.[9] Both SCLC and large cell neuroendocrine tumors (LCNEC) are categorized as high-grade neuroendocrine tumors.[11] In the present case, histological morphology and the positive rate of Ki-67 testing were different from those typical of SCLC or LCNEC, although neuroendocrine differentiation was observed.

The mechanism of histological transformation in *ALK* rearrangement-positive adenocarcinoma is not yet completely understood.[3] Loss of Rb and p53 function seems

to play an important role in transformation to SCLC in *ALK* rearrangement-positive lung cancer.[3] In epidermal growth factor receptor (*EGFR*)-mutant lung adenocarcinoma, inactivation of both Rb and p53 before treatment of EGFR-TKI is strong predictive factor of transformation to SCLC.[12] To our knowledge, three reports presently exist that investigate gene alterations by using next generation sequencing before and after transformation to SCLC in *ALK* rearrangement-positive NSCLC.[6-8] These case reports revealed that *p53*, *PTEN*, *Rb1*, and *NOTCH1* gene mutations, which are known to be involved in proliferation and carcinogenesis, were observed in tumor samples after transformation of the tumor.[6-8] Among all of these cases, ALK was retained after transformation. In the present case, immunohistochemical analysis showed that expression of Rb was positive and p53 was wild type both before and after treatment with alectinib. These findings suggest that factors other than Rb or p53 are required for transformation to neuroendocrine carcinoma in *ALK* rearrangement-positive NSCLC. There have been no report showing the precise role of cancer stem-like cells in transformation of ALK rearrangement-positive lung cancer. We are working on the new project to investigate its role in transformation of ALK rearrangement-positive lung cancer.

In the present case, CD133 and BCL-2 immunohistochemically became positive,

and SOX2 expression became strong after histological transformation. As a cell surface protein, CD133 is recognized as a marker of cancer stem-like cells.[13] Cancer stem-like cells are defined as cells within a tumor that possess the capacity to self-renew and generate the heterogeneous lineages of cancer cells that comprise the tumor.[14] Recent studies have revealed that the number of cancer stem-like cells increases after exposure to chemotherapy, and these cells are resistant to multiple anticancer drug treatments, and typically continue to grow.[13] Exposure to EGFR-TKIs reportedly also lead to the emergence of drug-tolerant cells that maintain variability, whereas the majority of the other cell populations typically die in *EGFR*-mutant NSCLC-derived cell lines.[15] We have previously shown that EGFR-TKI-induced drug-tolerant persisters were composed of two types of cells, cancer stem-like cells and senescent cells.[4] The expression of stem cell related markers such as oct3/4 and sox2, was high in cells with high expression of CD133, although we have not yet shown that CD133 directly play a role on histological transformation.[4] Cancer senescence represents a state of cell cycle arrest. Upregulation of Bcl-2 is reportedly required for the initiation of cell senescence in mouse melanoma cells.[16] In the present case, positive expression of BCL-2 after treatment with alectinib may suggest the occurrence of cell senescence. A previous study showed that SCLC transformation from *EGFR*-mutant cell lines was sensitive to a BCL-2 inhibitor, and

those lines were markedly more sensitive than EGFR-TKI-resistant NSCLC cell lines with the *EGFR* T790M resistance mutation.[17] Although the association of BCL-2 overexpression and LCNEC transformation in EGFR mutation-positive NSCLC seems to be fully clarified, BCL-2 may be a possible key molecule of this transformation.[18] These findings may indicate that CD133 and BCL-2 play important roles in histological transformation.

As a transcription factor, SOX2 plays an important role in the maintenance of stemness and tumorigenic activity in lung adenocarcinoma.[19] Overexpression of SOX2 can be detected in 71% of neuroendocrine carcinomas, whereas it is typically detected in 10% to 50% of adenocarcinomas.[20] A previous study reported that overexpression of SOX2 leads to a substantial increase in the number of committed progenitor cells, including neuroendocrine cells in mice, indicating that SOX2 may induce neuroendocrine differentiation in pluripotent cells.[21] Thus, SOX2 may be associated with neuroendocrine differentiation. Taken together, these immunohistochemical findings suggest that cancer stem-like cells, the number of which becomes increased during TKI therapy, might differentiate to neuroendocrine carcinoma under the influence of some differentiation regulators.

Histological transformation is more likely to occur because the morphology was

absolutely different between before and after treatment of ALK-TKI. But it is not completely denied that neuroendocrine component existed before treatment of alectinib because the small samples obtained by transbronchial lung biopsy could not represent entire picture of the tumor.

In summary, we showed that histological transformation induced by ALK-TKI was associated with cancer stem-like cells in *ALK* rearrangement-positive lung cancer. These findings could be important to clarifying the mechanism of transformation. Further studies are needed to investigate the association between cancer stemness and histological transformation in *ALK* rearrangement-positive NSCLC.

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Disclosure statement

None declared.

Author Contribution

N.K. and T.N. were responsible for conception and design. N.J. and T.I. performed pathological examination. Ki.K., N.K., N.J., M.T., D.T., K.N., T.N., M.Y., H.K. Ka.K. and Y.N. contributed to the analysis of data. Ki.K. and N.K. wrote the manuscript. All authors reviewed and approved the final manuscript.

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Figure legends

Figure 1 (**a**) Chest computed tomography (CT) scan sowing a tumor with diameter of 31 mm in the left lower lobe (arrow head). (**b**) Chest CT scan showing the primary tumor with a reduction in size after 3 months of treatment with alectinib (arrow head). (**c**) Chest CT scan showing enlarged mediastinal lymph nodes after 8 months of treatment with alectinib (arrow). (**d**) Chest CT scan performed after four cycles of chemotherapy with cisplatin–irinotecan combination therapy showing a reduction in size of the mediastinal lymph nodes (arrow).

Figure 2 Histopathological examination of (**a–d**) primary tumor before treatment with alectinib and (**e**–**h**) newly enlarged subcarinal lymph node after treatment with alectinib. (**a**) Hematoxylin and eosin (H&E) staining showing well- to moderately-differentiated adenocarcinoma, showing papillary structure or glandular formation. Immunohistochemical analysis showed positive results for (**b**) thyroid transcription factor 1 (TTF-1) and (**c**) anaplastic lymphoma kinase (ALK), and negative for (**d**) insulinoma-associated protein 1 (INSM1). (**e**) After treatment with alectinib, H&E staining shows poorly differentiated carcinoma and polygonal tumor cells with abundant cytoplasm composed of solid sheets or nests without papillary or acinar growth. Immunohistochemical analysis yielded positive results for (**f**) TTF-1 (weekly positive),

(**g**) ALK, and (**h**) INSM1.

Figure 3 Histopathological examination of (**a**–**c**) primary tumor before treatment with alectinib, and (**d**–**f**) newly enlarged subcarinal lymph node after treatment with alectinib. Immunohistochemical analysis yielded negative results for (**a**) BCL-2 and (**b**) CD133, and positive results for (**c**) SOX2 before treatment with alectinib; and positive results for (**d**) BCL-2 and (**e**) CD133 after treatment with alectinib. (**f**) SOX2 expression became strongly positive after treatment with alectinib.

Figure 4 Histopathological examination of (**a**, **b**) primary tumor before treatment with alectinib, and (**c**, **d**) newly enlarged subcarinal lymph node after treatment with alectinib. Immunohistochemical analysis yielded wild type results for (**a**, **c**) p53, and positive results for (**b**, **d**) retinoblastoma protein (Rb), both before and after treatment with alectinib.

Figure 5 Break-apart fluorescent in-situ hybridization (FISH) of specimens of the newly enlarged subcarinal lymph node after treatment with alectinib, showing rearrangement of the ALK gene (arrows: translocation-positive).

Figure1

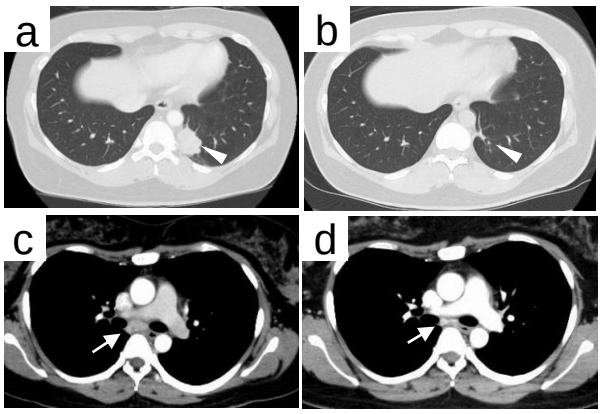

Figure 2 revised

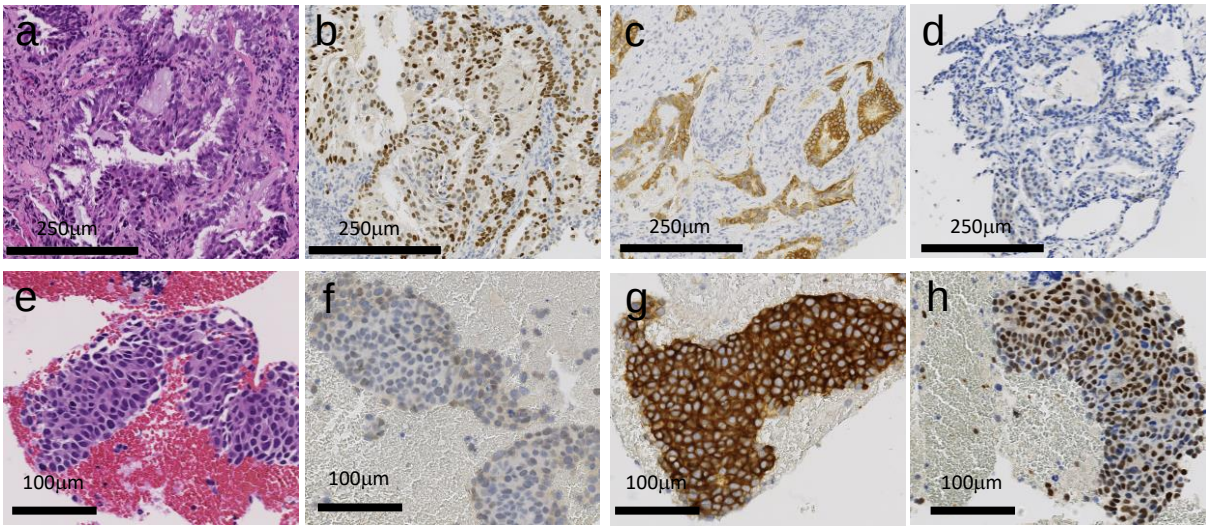

Figure 3
revised

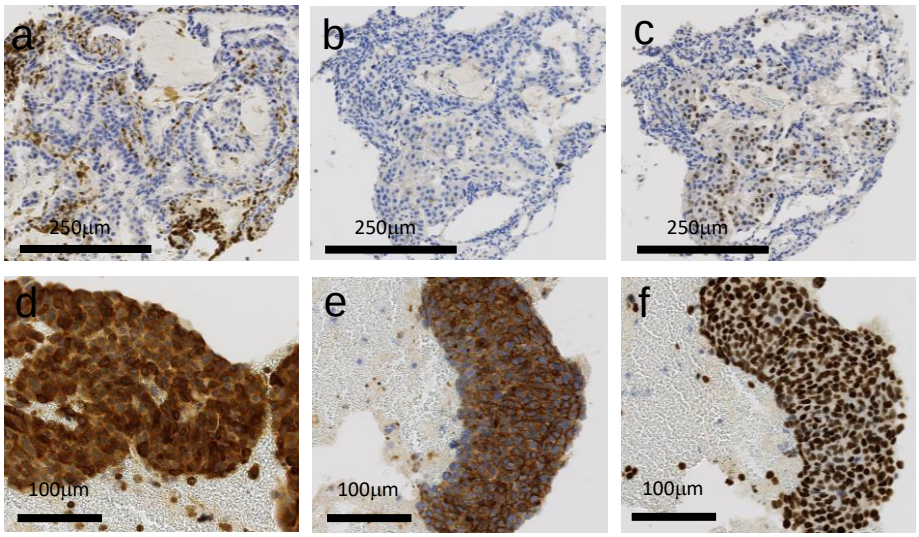

Figure 4

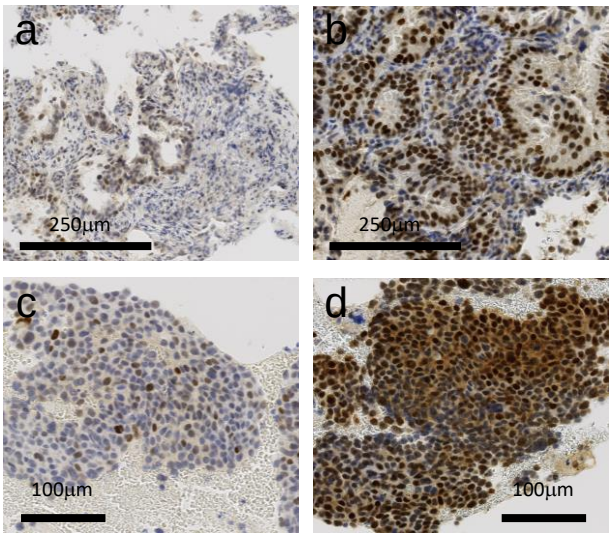

Figure 5