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ALK gene translocations and amplifications in brain metastases of non-small cell lung cancer

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ABSTRACT

Background: Increased incidence of brain metastases (BM) in non-small cell lung cancer (NSCLC) with *ALK* translocations was postulated, however, *ALK* gene aberrations in NSCLC-BM have not been investigated so far.
Methods: We investigated *ALK* and *EML4* gene aberrations (amplifications, translocations, inversions) by fluorescent in situ hybridization (FISH) ($n = 175$) and ALK and EML4 protein expression by immunohistochemistry ($n = 221$) in NSCLC BM and corresponding primary tumors.
Results: ALK translocations were found in 4/151 (2.6%; 3 of them involving *EML4*) of BM of adenocarcinomas (AC), 1/9 (11.1%) of adenosquamous carcinomas (ASC), 0/5 of squamous cell carcinomas (SCC) and 0/10 of large cell carcinomas (LCC). Rearrangement of *ALK* without involvement of *EML4* was seen in 1 AC-BM and rearrangement of *EML4* without involvement of *ALK* in 3 AC-BM, 1 ASC-BM and 1 LCC. *ALK* amplifications without gene rearrangements were found in BM of 16/151 (10.6%) AC, 2/5 (40%) SCC, 0/9 ASC and one LCC. *ALK* translocation status was constant between BM and primary tumors in 16 evaluable cases including two cases with *ALK–EML4* translocations Among these 16 cases *ALK* amplification was seen in two BM and none of the primary tumors. All cases with translocations but not with amplifications of *ALK* showed protein expression. We found no association of *ALK* gene status with patient age, gender or overall survival time.
Conclusions: ALK translocations and amplifications are found in approximately 3% and 11% of NSCLC-BM, respectively. While *ALK* translocations appear to be constant between primary tumors and BM, amplifications seem to be more prevalent in BM. *ALK* translocation, but not *ALK* amplification is associated with ALK protein overexpression. Further studies are needed to determine whether NSCLC-BM patients with *ALK* gene aberrations may benefit from specific inhibitor therapy.

1. Introduction

Non-small cell lung cancer (NSCLC) is a leading cause of cancer-related mortality [1]. The prognosis of patients with metastatic disease is poor with 5-year survival rates of approximately 2%. In the metastatic setting, systemic therapy with cytotoxic agents is standard for most patients. However, recent advances have shown that patient subpopulations whose tumors harbor distinct

Abbreviations: IHC, immunohistochemistry; FISH, fluorescent in situ hybridization.

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molecular alterations may benefit from specific targeted drugs. Among the most relevant "druggable" alterations besides activating *epithelial growth factor receptor* (*EGFR*) mutations to date in NSCLC are aberrations of the *anaplastic lymphoma kinase* (*ALK*) gene [2–5]. *ALK* translocations are present in approximately 2–5% of patients with NSCLC and are more common in tumors of younger patients, patients with a history of no or only light smoking and in adenocarcinomas. The most common translocation results in an aberrant fusion of *ALK* with the *microtubule-associated protein-like 4* gene (*EML4*), but other fusion partner genes of *ALK* have also been described (e.g. *TFG*, *KIF5B*) and differential responsiveness to ALK inhibition has been reported for such variants [6]. Activating *ALK* translocations lead to constitutive activation of a cytoplasmic chimeric protein that induces pro-neoplastic downstream signaling via several interconnected pathways such as the RAS/ERK and the PI3K/Akt cascade. NSCLC with ALK activating *ALK* translocation has been shown to be sensitive to treatment with the ALK inhibitor crizotinib and this drug has been approved in several countries [2–5].

Brain metastases (BM) are a common complication of patients with NSCLC and NSCLC is one of the most common causes of BM [7]. The occurrence of BM is associated with poor prognosis and high morbidity. Treatment options are generally limited and comprise neurosurgical resection, radiosurgery, radiotherapy and symptomatic therapy (e.g. corticosteroids, anticonvulsants). The role of systemic therapies including targeted agents in patients with BM is unclear due to the lack of adequately powered clinical trials. Drug treatment of BM is complicated by insufficient penetration through the blood–brain barrier (BBB) of several cytotoxic drugs. However, targeted drugs with adequate BBB penetration may be effective in patient with BM [8,9]. In NSCLC, several small studies and case reports indicate that epithelial growth factor receptor (EGFR) inhibitors may be beneficial in BM patients, especially in the presence of activating *EGFR* mutations [10–12]. To the best of our knowledge, the activity of ALK inhibitors has not been systematically investigated so far in patients with NSCLC-BM.

The mechanisms involved in BM formation have been only incompletely elucidated and no parameters reliable predicting risk of BM formation in NSCLC have been identified. Evidence from other tumor types shows that distinct molecular tumor phenotypes may associate with an increased risk for BM [13]. In NSCLC, an increased risk for BM has been related to some clinic-pathological and molecular factors such as advanced disease stage, large primary tumor size, or non-squamous histology and activation of the canonical WNT/TCF pathway [7,14]. Interestingly, one recent study indicated that *ALK* translocations may predispose to BM formation [15]. In contrast, another study found no association of *ALK* gene status with brain-metastatic behavior [16]. We undertook the present study to determine the frequency of *ALK* gene aberrations in BM of NSCLC in order to provide information on their potential role in BM formation and their relevance as therapeutic target in NSCLC-BM patients.

2. Patients and methods

2.1. Patients

All patients who had undergone surgery for brain metastases of NSCLC at the Department of Neurosurgery, Medical University of Vienna, Austria, between March 1990 and February 2011 were eligible for this study. In the vast majority of patients, a histologically confirmed primary lung cancer was evident. Brain metastases without histologically verified primary lung tumor, but adenocarcinoma morphology and TTF-1 positivity (lacking morphological features of a thyroid carcinoma) were also regarded as originating from NSCLC cancer. Clinical data and death dates were retrieved by chart review and from the databases of National Cancer Registry of Austria and the Austrian Brain Tumor Registry [17]. The study was approved by the ethics committee of the Medical University of Vienna.

2.2. Tissue micro arrays

We prepared a standard hematoxylin and eosin stained slide of each routinely formalin-fixed and paraffin-embedded tissue block of all patient's BM and, where available, primary tumor specimens and selected 2 tissue separate areas with representative and viable tumor tissue. We used a needle with a diameter of 1.5 mm to punch the selected 2 tissue areas per block and arranged all punch biopsy samples in paraffin blocks (tissue micro arrays), as described elsewhere [18,19]. For all fluorescent in situ hybridization (FISH) and immunohistochemical investigations 3–5 μm thick sections were cut from the tissue micro arrays.

2.3. Fluorescent in situ hybridization (FISH)

ALK gene status was evaluated by FISH using a commercially available triple color break apart single fusion probe (ZytoLight® SPEC ALK/EML4 TriCheckTM, ZytoVision, Bremerhaven, Germany). This probe set comprises two probes (orange and green) flanking the breakpoint cluster region of *ALK* gene and another probe (blue) covering the complete *EML4* gene including the breakpoint cluster region of *EML4*. So rearrangements (including translocations, inversions, and amplifications) of *ALK* and *EML4* genes can be detected. FISH was performed and analyzed according to the manufacturer's instructions, hundred cells were analyzed in each case. *ALK* rearrangements were diagnosed when more than 20 cells showed unequivocal translocations or inversions. As cutoff for amplification, ≥6 signal copies per cell were chosen.

2.4. Immunohistochemistry

ALK protein expression was investigated immunohistochemically with a benchmark Ultra Immunostainer (Ventana, Tucson, AZ) [20]. The staining procedure included pretreatment with cell conditioner 1 (pH 8, Ventanta) for 25 min and incubation with UV inhibitor (Ventana) for 4 min, followed by incubation with the anti-ALK antibody (clone D5F3, kindly donated by Cell Signaling Technology, Danvers) at 36 °C for 120 min and a dilution of 1:250. Antibody incubation was followed by 8 min incubation with Amplifier A (Ventana), 8 min incubation with Amplifier B (Ventana), 8 min incubation with UV HRP UNIV MULT (Ventana), 8 min incubation with UV DAB (Ventana) and UV DAB H2O2 (Ventana), 4 min incubation with UV Copper (Ventana) and counterstaining with one drop of hematoxylin (Ventana) for 4 min and one drop of bluing reagent (Ventana) for 4 min.

A specimen was considered as positive if >50% of tumor cells showed distinct cytoplasmic expression. A sample of an anaplastic large cell lymphoma with known *ALK* translocation served as positive control.

EML4 was detected with two different antibodies directed against the C-, and the N-terminal end of EML4, respectively: EML4 monoclonal antibody (M01), clone 3C10 against the 5′-end (Abcam, Cambridge, UK, 1:100); and antibody NBP1-86805 against the 3′-end (Novus Biologicals, Littleton, CO, 1:2000) of EML4.

2.5. Statistics

Survival was calculated from the day of surgery for BM until death of a patients using log rank test. Loss to follow up was considered as censored observation. A two tailed *p*-value of equal or