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Amplification but not translocation of *anaplastic lymphoma kinase* is a frequent event in oesophageal cancer

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Abstract ***Introduction:*** Translocations of *anaplastic lymphoma kinase* (*ALK*) to various fusions partners and formation of oncogenic fusions proteins have been demonstrated in a variety of human malignancies. These fusion-proteins are potential pharmaceutically targets. Aim of this study was to investigate *ALK* gene status in a large cohort of squamous cell carcinoma (SCC) and adenocarcinoma (AC) of the oesophagus.
Materials and Methods: 117 SCCs and 136 ACs were included into this study. *ALK* and *EML4* gene status were evaluated by fluorescence in situ hybridisation (FISH) using a triple colour break apart single fusion probe and a probe against 2p11. ALK and EML4 protein expression was determined by immunohistochemistry. Data on expression of ALK downstream effector tyrosine-705 phosphorylated STAT3 (pSTAT3) were available from a previous study.
Results: FISH was performed successfully in 251 cases. All cases were negative for *ALK* translocations, while 14/135 (12.1%) of SCCs and 14/116 (10.4%) of ACs showed *ALK* amplifications. Concomitant *EML4* amplifications were present in 27/28 cases with *ALK* amplifications. Three cases (two SCC, one with additional *ALK* & *EML4* amplification and one AC) showed *EML4* translocations not involving *ALK*. None of the tumours with *ALK* amplification showed ALK protein expression, and no correlation with clinical parameters, survival or pSTAT3 expression was observed.
Conclusions: While *ALK* translocations are not present in oesophageal cancer, *ALK* amplifications are common events with comparable rates in SCC and AC. Since *ALK* amplified breast cancer cells were shown to respond to *ALK* inhibitors, *ALK* amplified oesophageal cancers might be considered as possible candidates for therapies targeting *ALK*.

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1. Introduction

Anaplastic lymphoma kinase (ALK) is a tyrosin kinase receptor playing an important role in the development of the brain.[1] *ALK* translocations and formation of oncogenic fusions proteins have been demonstrated in a variety of human malignancies, as anaplastic large cell lymphoma[2] and non-small cell lung cancer (NSCLC).[3] *ALK* mediated activation of STAT3 by phosphorylation seems to play an important role in the oncogenic effects of *ALK*.[4,5] Oncogenic *ALK* effects are of great interest since ALK fusion proteins can be inhibited.[6,7]

Oesophageal cancer is a common tumour worldwide, and characterised by a high rate of recurrency and poor prognosis in most cases. Generally two subtypes of this cancer entity exist: squamous cell carcinoma (SCC), which is associated with smoking and alcohol consumption, and adenocarcinoma (AC), which is strongly associated with chronic gastroesophageal reflux disease.[8] Therapeutic opportunities are limited especially in advanced stage disease making the search for new therapeutic targets of high relevance. Only few data obtained by proteomics on ALK in oesophageal SCC exist, indicating that ALK fusion protein might play a role also in this disease, while no data in oesophageal AC exist.[9,10]

Aim of this study was to investigate *ALK* gene status and protein expression in a large cohort of oesophageal SCCs and ACs.

2. Materials and methods

All patients who had undergone primary surgery for oesophageal cancer at the Department of Surgery of the Medical University of Vienna between the years 1992 and 2010 were eligible for this study. From all patients where sufficient tumour tissue was available ($n = 253$), formalin fixed, paraffin embedded tissue was cut into 4m thick histological slides.

2.1. Fluorescence in situ hybridisation (FISH)

ALK gene status was evaluated by FISH using a triple colour break apart single fusion probe (ZytoLight® SPEC ALK/EML4 TriCheck™, ZytoVision, Bremerhaven, Germany). This probe set comprises two probes (orange and green) flanking the breakpoint cluster region of *ALK* and one probe (blue) covering the complete *echinoderm microtubule associated protein like 4* (*EML4*) gene including the breakpoint cluster region of *EML4* (Fig. 1). Consequently, rearrangements (including translocations, inversions and amplifications) of *ALK* and *EML4* can be detected. FISH was performed and analysed according to the manufacturer's instructions. Signals were interpreted according to the instructions. Hundred cells were analysed in each case. As cutoff for amplification, $\geqslant 8$ signal copies per cell were chosen to avoid false positive results in cases with tetraploid karyotypes. In addition, in cases with $\geqslant 8$ *ALK* copies, chromosome 2 was investigated using a FISH probe against 2q11.2 (Zytolight® SPEC 2q11 probe, ZytoVision, Bremerhaven, Germany). The ratio between *ALK* and 2p11 signals was calculated, and in analogy to determination of *HER2* status, a result of $\geqslant 2$ was considered as true gene amplification.[11]

2.2. Immunohistochemistry

ALK protein expression was investigated immunohistochemically with a benchmark Ultra Immostainer (Ventana, Tucson, AZ) using three antibodies, comprising CONFIRM ALK01 (Ventana, ready to use), NCL-ALK (Novocastra, Lecia Microsystems, Wetzlar, Germany, 1:30), and ALK D5F3 (Cell Signaling Technology, Danvers, MA, 1:250).[12] 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, detecting aminoacids 1–63 of human EML4 (Abcam, Cambridge, UK, 1:100); and antibody NBP1-86805, covering in EML4-001 amino acids 857–942, in EML4-002: amino acids 799–844, in EML4-003: amino acids 868–953 and in EML4-201: amino acids 121–206 (Novus Biologicals, Littleton, CO, 1:2000).

Data on immunohistochemically assessed tyrosine-705 phosphorylated STAT3 (pSTAT3) were available from a previous study.[13]

3. Results

FISH was successful in 251 patients (135 AC, 116 SCC). Pathohistological grading and staging of patients are provided in Table 1. Mean observation time was 55 ± 5 (standard error) months. During this period of time, 136 (54.2%) patients developed recurrent disease, and 117 (46.6%) died of their cancer. Aberrations were detected in 30/251 cases (15/116 SSC cases and 15/135 AC cases) and comprised amplifications and translocations.

3.1. Amplifications are more frequent than translocations

Twenty-eight cases revealed gene amplifications (14 SSC cases (12.1%), 14 AC cases (10.4%).Among the 28 cases with amplifications, seven cases (five SCCs, two ACs) demonstrated amplification patterns restricted to a distinct part of the cell suggesting ring chromosomes or homogenously staining regions as putative amplification mechanisms in these cases (Fig. 1A1 and A2). In 27