Status of Anaplastic Lymphoma Kinase (ALK) in Malignant Mesothelioma

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Abstract. *Background: Malignant mesothelioma (MM) is a particularly aggressive type of primary tumor, associated with exposure to asbestos, and characterized by high mortality. To date, there is no curative therapy for MM. The receptor anaplastic lymphoma kinase (ALK) was found to be mutated in many cases of cancer and used as a target in biological therapies. We investigated whether this pharmacological treatment could also be applicable to MM. Materials and Methods: The state of ALK was analyzed by immunohistochemistry and fluorescent in situ hybridization in 63 MM tissue specimens. Results: None of the 63 MM samples showed overexpression or translocation of ALK. Conclusion: Our preliminary data exclude the utility of analysis of the ALK gene in MM and suggest that ALK inhibitor therapy is not applicable to MM.*

Malignant mesothelioma (MM) is a particularly aggressive type of primary tumor, associated with exposure to asbestos and characterized by high mortality. Pleural MM (MPM) is the most common form. It has been estimated that in the next 15-20 years in Western Europe the number of deaths from MPM will increase from 5,000 in 1998 to 9,000 in 2018 (1). In Italy, a peak of 800 deaths per year is expected between 2012 and 2024 (2).

MPM is a rare tumor type: its incidence in the Italian population is 3.16 and 1.12 cases in men and women per 100,000 inhabitants (2). In the provinces of Genova and La Spezia, the incidence of MPM is the highest in the world (about 12 cases per 100,000 inhabitants) (3).

To date, there is no curative therapy for MM; rather a multidisciplinary approach, involving the use of surgery, chemotherapy and radiotherapy, aims to alleviate the symptoms and lengthen survival. Survival tends to be 11 to 12 months after diagnosis (4).

Therefore, there is an urgent need for an effective therapy, such as the innovative targeted therapies, namely biological therapies selective only against tumor cells, obtaining a reduction of side-effects and greater clinical results.

The anaplastic lymphoma tyrosine kinase (ALK) receptor was found to be re-arranged, translocated or deleted in many tumor types, including non-small cell lung cancer (NSCLC), which represents approximately 80-85% of all lung cancers (5).

Recently, several inhibitory molecules of the kinase activity of ALK have been described. One of these molecules is crizotinib, approved by the Food and Drug Administration as a drug indicated in the treatment of patients with locally-advanced or metastatic ALK-positive NSCLC (6). Crizotinib not only inhibits ALK, but also other tyrosine kinase receptors such as the hepatocyte growth factor receptor (HGFR, c-MET) and receptor of origin Nantais (RON) (6).

ALK is a tyrosine kinase receptor physiologically-expressed in specific regions of the central and peripheral nervous systems (7). Its most frequent chromosomal aberration is the translocation re-arrangement that involves the gene echinoderm microtubule-associated protein-like 4 (*EML4*); the result of this aberration is the fusion of the two genes and the consequent formation of a fusion protein EML4–ALK that is constitutively active, with an important role in the control of cellular proliferation (7). The *EML4–ALK* translocation is rather rare, in fact it is reported in the literature as being present in up to a maximum of 7% of all NSCLC (8-11).

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In addition to NSCLC, re-arrangements of *ALK* have been found in many other cancers, such as anaplastic large cell lymphomas, neuroblastomas and inflammatory myofibroblastic tumors (12), but there are no data regarding MM.

Crizotinib is currently used for the treatment of advanced NSCLC and in patients carrying the translocation of *ALK*, showing a clinical benefit in 80-90% of cases and a median progression-free survival of 9-10 months (8). The same therapeutic approach may also be appropriate for MM.

In the present study, we assessed the status of ALK protein expression by immunohistochemistry (IHC) and the presence of *EML4–ALK* translocation by fluorescent *in situ* hybridization (FISH) in order to evaluate the eligibility of patients with MM for the therapy with the crizotinib.

To our knowledge, this is the first report on the analysis of the state of ALK in MM.

Materials and Methods

Specimen characteristics. We analyzed a total of 63 MM (from 43 males and 20 females) in different stages and organ location (Table I). The formalin-fixed and paraffin-embedded tissues were derived as follows: 30 cases of MM from the tissue microarray MS801 (US Biomax Inc, Rockville, MD, USA), 13 cases of MM from the Division of Histopathology and Cytopathology, National Institute for Cancer Research in Genova, Italy, and 20 cases of MM from the Division of Histopathology and Cytopathology, Azienda Sanitaria Locale n˚ 5 in La Spezia, Italy. All the patients were hospitalized in the period between 2006 and 2013.

The positive control was represented by NSCLC cases submitted for routine analysis to the Division of Histopathology and Cytopathology, National Institute for Cancer Research,Genova. Five cases of normal mesothelial tissues from the same tissue microarray were used as negative controls.

No ethics approval was required for this study. The study was performed in line with the Helsinky Declaretion and our institutional regulations. All patients provided informed consent for participant and anonymous publication of data.

Immunohistochemistry. The IHC was carried out by an automatic immunostainer Ventana Benchmarck XT (Ventana Medical System Inc, Tucson, AZ, USA) according to the established protocol.

The state of ALK protein was analyzed with IHC by means of the anti-ALK antibody (mouse monoclonal, clone anti-ALK01, Roche) pre-diluted and incubated at 37˚C for 2 hours. Antigens were localized by means of an ultra-view universal DAB Detection kit (Ventana Medical Systems) based on the polymeric biotin-free system, a particularly sensitive indirect system.

Counterstaining was obtained with Gill's hematoxylin (Ventana Medical Systems Inc) incubated for 8 minutes at room temperature, followed by incubation for a few minutes with Bluing-reagent (Ventana Medical Systems Inc), a solution that increases the contrast of the hematoxylin towards a blue color. Once the reaction ended, the slides were manually mounted with Eukitt Mounting Medium (Bioptica, Milano, Italy).

The IHC reaction showed the fusion protein EML4–ALK, which displayed mainly a pattern of membrane expression and, to a lower extent, of cytoplasmic expression.

Table I. *Characteristics of patients*.

Parameters	N (%)
Patients	63
Age, years	
Median	60
Range	5-85
Gender	
Female	20 (31.74)
Male	43 (68.25)
Histology	
Epithelioid,	36 (57.14)
Sarcomatoid	13 (20.63)
Biphasic	9 (14.28)
Papillary	3 (4.76)
Desmoplastic	2 (3.17)
TNM	
T1N0M0	10 (15.87)
T2N0MO	9 (14.28)
T3/T4N0M0	2 (3.17)
Unknown	42 (66.66)
Tissue	
Pleura	41 (65.07)
Abdominal cavity	8 (12.69)
Cardiac pericardium	5 (7.93)
Lung	3 (4.76)
Other*	6 (9.52)

*Bone (n=1), blood vessel (n=1), mesentery (n=1), omentum (n=1), mediastinum (n=1), retroperitoneum (n=1).

The reaction was assessed independently by two pathologists and conflicting results were re-evaluated together.

FISH. To perform FISH, all samples of formalin-fixed and paraffin-embedded tissues were subjected to pretreatment and protease treatment by means of Paraffin IV Pre & Post Hybridization Wash Buffer Kit (Abbott, Milano, Italy). ALK was analyzed using the FISH probe specific to the ALK locus (Vysis LSI ALK dual-color break apart rearrangement probe; Abbott Molecular, IL, USA).

The results were viewed by fluorescence microscopy. For each sample, the number of signals was counted in almost 50 nuclei per slide. The hybridization of the samples and interpretation of the results were performed following the guidelines from the manufacturer. In cases of wild-type *ALK*, the FISH showed fused red and green signals as a unique yellow signal, or as two distinct signals, red and green, close to one another. In contrast, in the case of translocation, the signals were separated by a gap, or the nuclei were without green signals. Signals separated by two or more signal diameters were considered split. The reactions were assessed independently by two pathologists and conflicting results were re-evaluated together.

Results

By previously described IHC and FISH methods, we evaluated the status of *ALK* in a total of 63 MM tissues including 36 epithelioid, 13 sarcomatoid, 9 biphasic, 3 papillary and 2 desmoplastic types (Table I).

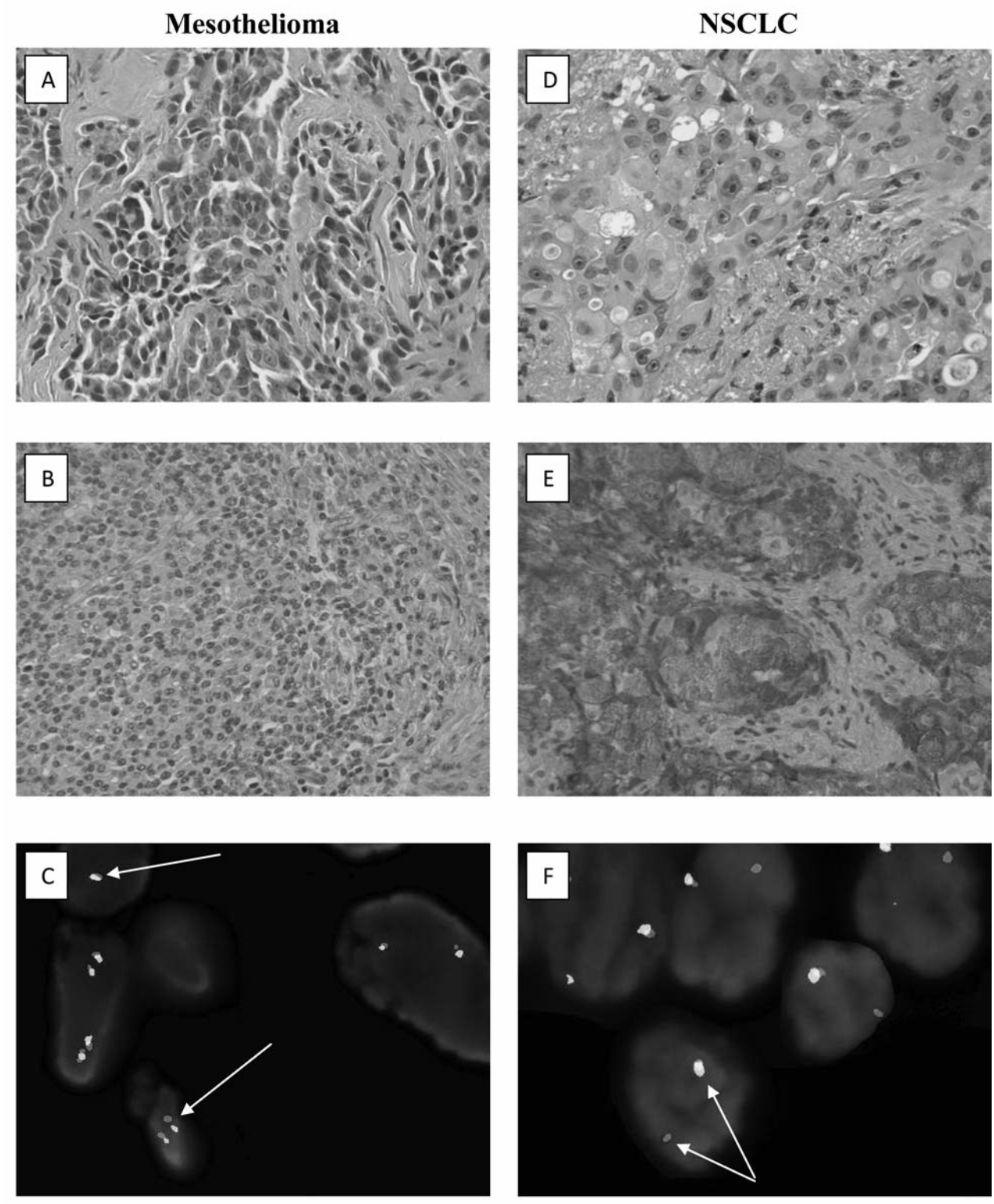

Figure 1. *Representative case of ALK expression and gene status in a sample of epthelioid mesothelioma (A, B, C) and non-small cell lung cancer positive control (D, E, F). A, D: Hematoxylin-eosin (×40); B, E: immunohistochemistry by antibody to ALK (×40); C, F: fluorescent in situ hybridization (×100) by probe specific for the ALK locus. Wild-type sample, such as mesothelioma, exhibited a unique signal or close distinct signals (white and gray as indicated by arrows in C). Specimens, such as the non-small cell lung cancer shown, were considered positive for ALK rearrangement when more than 15% of tumor cells demonstrated distant split signals (white and gray as indicated by arrows in F).*

All the 63 MM samples were negative for the overexpression of ALK protein and did not exhibit genetic abnormalities (Figure 1). In contrast, one specimen of NSCLC showed *ALK* translocation and overexpression of ALK protein (Figure 1).

Discussion

Despite advances in chemotherapy, radiation therapy and surgical management, the prognosis of patients with MM remains poor, with a median survival after diagnosis of less than one year (4). Today, there is a strong incentive to develop new therapeutic modalities, as well as targeted therapies already used with benefit for many tumors (12). Targeted therapies are able to inhibit the specific target considered causative of the neoplastic transformation in an efficient manner and with low toxicity. Specific genetic abnormalities are predictive of the appropriate use of targeted-therapy (6).

In the present study, we analyzed the *ALK* status in patients with MM to determine their eligibility for ALK inhibitor therapy with crizotinib. To our knowledge, there are no data in the literature referring to such an approach.

In NSCLC specimens, the diagnostic criteria normally used to evaluate if the patient is suitable for therapy with crizotinib rely on the detection of the translocation *EML4-ALK* by FISH, a molecular biological technique that uses fluorescent probes complementary to gene sequences of interest. The FDA approves the use of crizotinib on the basis of a positive FISH test; thus, for this reason, FISH remains the gold standard technique for ALK status investigation (13, 14).

In addition to FISH, we evaluated the samples by IHC, a technique usually used in pathology to detect the presence of proteins by means of specific antibodies. IHC still cannot be used alone for an indirect detection of *ALK* translocation, but must always be associated with FISH. Generally, there is concordance between IHC and FISH results (13, 14). Therefore, to detect the status of ALK, at both the protein and genetic level in MM, we applied both techniques.

Our preliminary data revealed MM to be negative for the overexpression of ALK protein and for genetic abnormalities (translocation, amplification). Thus, our data strongly suggest that ALK-inhibitory therapy with crizotinib is not applicable to MM. We cannot exclude that, in a very small percentage of cases, it is possible to find a genetic abnormality of *ALK* or its overexpression. However, we believe that the cost–benefit ratio for such determination would be very high and this approach should be carefully evaluated. Moreover, further investigation for other genetic abnormalities in MM are needed in order to assess new cytotoxic agents and targeted drugs.

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