ALK expression favorably impacts the prognosis of *NRAS*-mutated metastatic melanomas

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Abstract. Recent studies reported the expression of anaplastic lymphoma kinase (ALK) in malignant melanomas. The aim of this study was to investigate whether ALK expression is associated with specific clinical and molecular characteristics of melanoma metastases, and to evaluate its correlation with survival outcomes. Seventy-one patients with metastatic melanoma were investigated. Clinical features and survival outcomes were analyzed and correlated to ALK expression, as detected by immunohistochemistry and reverse transcription-quantitative polymerase chain reaction, and to the mutational status of *BRAF*, *KRAS*, *NRAS*, and *PIK3CA*. No translocations or ALK alternative isoforms were identified. ALK expression was mainly detected in *NRAS* mutated metastatic lesions. Interestingly, among *NRAS*-mutated patients, ALK positive samples displayed a significantly more favorable outcome in terms of disease specific survival, as compared to ALK negative ones. In conclusion, we suggest that ALK positive/*NRAS* mutated metastases represent a specific subset of metastatic melanomas, associated with a better prognosis. Validation of these observations in larger cohorts could contribute to understand the molecular events cooperating to melanoma progression, in addition to open new perspectives in the clinical and therapeutic management of this subgroup of patients.

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Introduction

Anaplastic lymphoma kinase (ALK) is a tyrosine kinase receptor frequently rearranged, mutated, or amplified in specific neoplastic diseases, including lymphoma, neuroblastoma, non-small cell lung cancer, and to a lesser extent in melanoma (1). In addition, ALK-specific mRNA and protein have been described in several cell lines from solid tumors of ectodermal origin, including melanoma (2). *ALK* break points have been identified in four acral cases (6.9%) of acral/mucosal melanomas from southern China (3). More recently, a novel ALK isoform derived from a *de novo* alternative transcription initiation (ATI) site in ALK intron 19 (ALK^{ATI}) has been described by Wiesner *et al* (4). Using the RNA-seq dataset of the TCGA project, these authors found ALK^{ATI} expression in 11% of melanoma patients (38/334) and sporadically in other human cancer types, but not in normal tissues (4). Thereafter, the same group identified ALK^{ATI} expression in 3% of 303 metastatic melanoma patients (5).

In the present study, we investigated the prognostic significance of ALK expression, as detected by immunohistochemistry and reverse transcription-quantitative polymerase chain reaction (RT-qPCR), in a cohort of metastatic melanomas characterized by *BRAF*, *KRAS*, *NRAS* and *PIK3CA* mutational status.

Materials and methods

Patients. A retrospective series of 71 metastatic melanoma patients with complete clinico-pathological information underwent mutational analyses at the Pathology Unit and were followed-up at the Dermatologic Clinic of 'Città della Salute e della Scienza' University Hospital (Torino, Italy). The study was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and within guidelines and regulations by the Research Ethics Committee of the University of Turin. Clinical, epidemiological and histological data were collected from the medical history of patients, all diagnosed, treated and followed-up according to previously reported protocols, after written informed consent (6-8).

Disease specific survival (DSS) was calculated from the date of primary lesion diagnosis to the date of patient death or last follow-up. Disease free interval (DFI) was calculated from the date of primary lesion diagnosis to the date of tumour progression/recurrence or last follow-up. Ethical approval for the present study was obtained from the Ethical Committee of our Institution.

Mutational status assessment. Metastatic tumor sections were submitted to DNA extraction as previously described (9). Mutational detection was performed using the Sequenom MassARRAY® system (Sequenom, San Diego, CA, USA) in conjunction with The Myriapod Colon Status kit that identifies 58, 54, 23 and 66 nucleotide substitutions in the *KRAS*, *NRAS*, *BRAF* and *PIK3CA* genes, respectively. Mutant and wild type alleles were discriminated using the Sequenom MassARRAY® Analyser 4 platform.

ALK immunostaining. Melanoma metastases used for the study were previously fixed in 4% buffered formaldehyde, routinely processed and paraffin embedded. For each case, three micrometer-thick paraffin sections were collected on superfrost plus slides and tested by immunohistochemistry using anti-ALK rabbit monoclonal antibody (clone D5F3; Ventana Medical Systems, Inc., Tucson, AZ, USA). ALK detection was performed on the fully automated Ventana BenchMark XT System using the recommended protocol. ALK immunostaining was evaluated by two independent pathologists applying a 4-tier (0-3) scoring scheme (negative: 0; mild cytoplasmic: 1; moderate smooth cytoplasmic: 2; intense granular cytoplasmic staining: 3) with either diffuse or focal pattern.

ALK transcript detection. Total RNA from formalin-fixed paraffin embedded (FFPE) samples were extracted using the miRNeasy FFPE Kit (QIAGEN), according to the manufacturer's protocols. cDNA was obtained from 0.2 μg of total RNA treated previously with RNase-free DNase (Promega Corporation, Madison, WI, USA) using reverse transcriptase SuperScript III (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and gene-specific reverse primers (Table I). RT-qPCR was performed with Thermal iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions. The PCR cycling conditions were as follows: 95˚C for 5 min, followed by 40 cycles at 94˚C for 10 sec and 60˚C for 30 sec. Oligonucleotide primer pairs used for RT-qPCR were designed with PrimerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to obtain amplicons of 70-110 bp (Table I). To confirm amplification specificity, PCR products were subjected to the analysis of melting curve, linearity, and slope of standard curve. PCR assays were performed in triplicate. Gene-expression results were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expressions and quantified using the ΔCt method, as previously described (10). Samples with GAPDH Ct value >30 were excluded from the analysis. Expression levels of ALK exons were compared to commercially available RNA from SH-SY5Y neuroblastoma cell line (Applied Biological Materials Inc., Richmond, BC, Canada), which expresses full length ALK transcripts (11,12). ALK primers used to verify the human origin of SH-SY5Y neuroblastoma cell line and detect full length ALK expression were designed according to the following ALK mRNA reference sequences: ENST00000389048.7 (human); ENSMUST00000086639.4 (murine) (13-15). Specifically, ALK1798R oligonucletiode and ALK1718F and ALK1790R primers were used for gene-specific retrotranscription (exon 3) and qPCR (exons 2-3), respectively (Table I).

Statistical analysis. Statistical analyses were performed using Stata/SE12.0 statistical software (STATA, College Station, TX, USA). $P<0.05$ was considered to indicate a statistically significant difference. Differences in ALK expression were analyzed using the χ^2 test and the Fisher's exact test for small numbers. Survival curves between different groups, according to ALK expression, were plotted using the Kaplan-Meier method and the statistical comparisons were performed with Log-rank test.

Results

The clinico-pathological features of the included cases are shown in Table II. Immunohistochemical analysis identified 10/71 (14%) ALK positive metastases (4 regional skin, 4 lymph node, 1 lung, 1 spleen metastasis sites), showing a predominantly cytoplasmic staining (Fig. 1A and B). Among these, 4 cases were scored 1+, 3 cases 2+, and 3 cases 3+. Transcriptional analysis by RT-qPCR using 5' and 3' exon specific primers detected the expression of full-length ALK mRNA in 8/10 ALK positive samples (Fig. 1C) and in 2/61 ALK negative samples, thus supporting the immunohistochemical observations (P<0,001). No significant imbalances of ALK exons expression suggestive of ALK fusions or ALK^{ATI} isoform were detected.

ALK expression was not associated with gender, age, or classical melanoma prognostic factors such as Breslow thickness, ulceration, or mitoses. *BRAF*, *KRAS*, *NRAS* and *PIK3CA* mutational analysis recognized 9/10 ALK positive samples as *NRAS* mutated, one *BRAF* mutated (score 3+ diffuse), and none *KRAS* or *PIK3CA* mutated, nor wild-type. Therefore, ALK expression was significantly ($p=<0.001$) enriched in *NRAS* mutated metastases (9 out of 22; Table II). No statistically significant difference in DFI or DSS was observed between ALK positive and negative patients overall. However, among *NRAS* mutated patients (11 *NRAS* Q61R, 11 *NRAS* Q61K), ALK positive samples (5 *NRAS* Q61R, 4 *NRAS* Q61K; Tables III and IV) displayed a significantly better DSS compared to ALK negatives ($P=0.050$; Fig. 1D and E). In *NRAS* mutated patients ALK expression was not correlated with other clinico-pathological variables.

Discussion

Melanoma represents the fifth most common tumour in humans and is considered one of the most invasive, therapy-resistant and metastatic malignancy, with only 10% of metastatic patients surviving 5 years post-diagnosis. In addition, over the past decades, its incidence has been increasing by 3-8% per year in Western countries (16,17). Therefore, a deeper

Table I. ALK gene-specific reverse primers.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Use
ALK Ex 2/3	ALK1718F CTGTCTCATCGCAGCCGATA	ALK1790R GTGGAGGGGAATACTCCAGC	RT
		ALK1798R GTCATGCAGTGGAGGGGAAT	RT-qPCR
ALK Ex 20/21	ALK4284F TGCCGCGGAAAAACATCACC	ALK4371R TTGGGCATTCCGGACACCTG	RT
		ALK4380R CTTGGGTCGTTGGGCATTC	RT-qPCR
ALK Ex 28/29	ALK5050F GCAACATCAGCCTGAAGACA	ALK5140R AGCGGTGTTGATTACATCCG	RT
		ALK5144R GCAAAGCGGTGTTGATTACA	RT-qPCR
HK	GAPDHF TCTTTTGCGTCGCCAGCCGAG	GAPDH150R TGACCAGGCGCCCAATACGAC	RT-qPCR

ALK, Anaplastic lymphoma kinase; GAPDH, (glyceraldehyde-3-phosphate dehydrogenase); F, forward; R, reverse; HK, housekeeping gene.

Table II. Clinical characteristics of patients across mutational status.

Characteristics	Total	WT (n=19)	*BRAF*-mutated (n=30)	*NRAS*-mutated (n=22)	P-value
Sex					
Female	29	8	14	7	0.556
Male	42	11	16	15	
Age, years, median (range)	62 (25-86)	66 (39-83)	53 (25-83)	67 (32-79)	0.002
Breslow, mean ± SD	3.67±2.61	4.26±2.57	3.44±2.95	3.33±1.96	0.500
Histotype					
SSM	35	7	16	12	0.749
NM	12	4	4	4	
Other[a]	24	8	10	6	
Ulceration					
Absent	53	14	20	19	0.271
Present	18	5	10	3	
Mitosis					
<1	36	8	14	14	0.328
≥1	35	11	16	8	
Stage at diagnosis					
I/II	37	11	18	8	0.206
III	27	8	10	9	
IV	7	0	2	5	
ALK IHC					
Negative	61	19	29	13	<0.001
Positive	10	0	1	9	

[a]Other histotypes include lentigo maligna melanoma, acral lentiginous melanoma, amelanotic melanoma or spitzoid melanoma. WT, wild type; F, female; M, male; SSM, superficial spreading melanoma; NM, nodular melanoma; IHC, immunohistochemistry; SD, standard deviation.

understanding of the molecular events regulating melanoma aggressiveness and metastatic dissemination is essential to develop new relevant biomarkers and therapeutic strategies.

In the present study, according to previous reports (5), we have documented that ALK protein expression can be detected by immunohistochemistry in a significant subset of

Table III. Clinical characteristics of NRAS mutated patients.

Characteristics	Total	ALK IHC negative	ALK IHC positive	P-value
Sex				0.899
Female	7	4	3	
Male	15	9	6	
Age, years, median (interval)	67 (32-79)	68 (32-78)	66 (46-79)	1.000
Breslow, mean ± SD	3.33±1.96	3.17±1.32	3.58±2.81	0.707
Histotype				0.868
SSM	12	7	5	
NM	4	2	2	
Other[a]	6	4	2	
Ulceration				0.271
Absent	19	12	7	
Present	3	1	2	
Mitosis				0.378
<1	14	9	5	
≥1	8	4	4	
Stage at diagnosis				0.542
II	8	4	4	
III	9	5	4	
IV	5	4	1	
First site of progression				0.683
Regional	18	11	7	
Distant	4	2	2	

[a]Other histotypes include lentigo maligna melanoma, acral lentiginous melanoma, amelanotic melanoma or spitzoid melanoma. F, female; M, male; SSM, superficial spreading melanoma; NM, nodular melanoma; IHC, immunohistochemistry; SD, standard deviation.

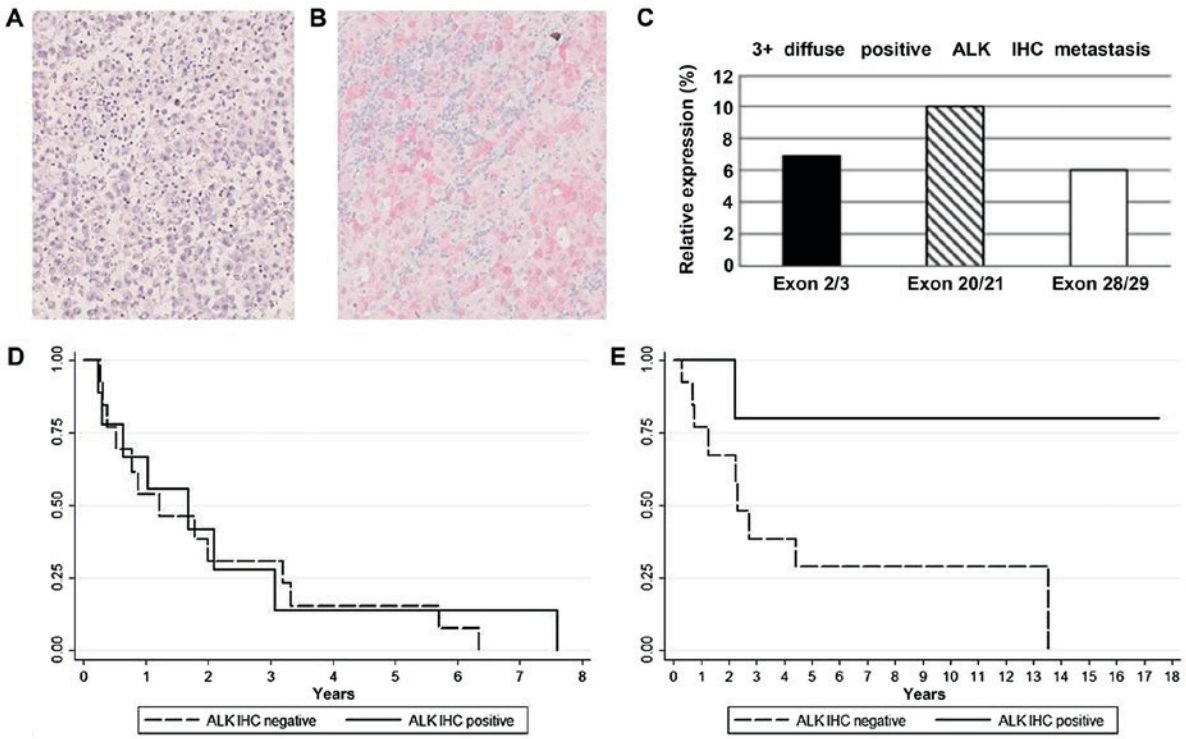

Figure 1. (A) Immunostaining of a representative ALK negative melanoma metastasis (magnification, x20); (B) Immunostaining of a representative ALK positive melanoma metastasis (magnification, x20); (C) Expression of full length ALK transcript as detected by RT-qPCR; (D) DFI and (E) DSS Kaplan-Meier curves in NRAS mutated patients on the basis of ALK expression. ALK, anaplastic lymphoma kinase; DFI, disease free interval; DSS, disease specific survival.

Table IV. Clinical and pathologic features of ALK-positive metastatic melanoma in NRAS mutated patients.

Case	Age	Sex	Site of metastasis	Site of primary	Breslow thickness	ALK IHC metastasis	RT-PCR	NRAS mutation	Therapy at first progression	Therapy at 2nd progression
1	47	M	Skin	Trunk	3,20	2+ focal	Positive	Q61K	Surgery alone	Ipilimumab
2	74	M	Lung	Arm	9,00	2+ focal	Positive	Q61K	Ipilimumab	-
3	74	F	Skin	Arm	1,60	1+ focal	Positive	Q61R	Surgery alone	-
4	66	M	LN	Leg	2,5	3+ diffuse	Positive	Q61K	Surgery alone	Anti-PD1
5	62	M	Skin	Back	3	2+ focal	Positive	Q61K	Surgery alone	Anti-PD1
6	77	F	LN	Leg	1,2	1+ focal	Negative	Q61R	Surgery alone	Electro-chemotherapy
7	46	M	Spleen	Trunk	3	1+ focal	Negative	Q61R	Surgery alone	-
8	61	M	LN	Head	1.3	1+ focal	Positive	Q61R	Surgery alone	-
9	79	F	LN	Back	3,50	3+ diffuse	Positive	Q61R	Surgery alone	Ipilimumab

F, female; M, male; LN, lymph node; IHC, immunohistochemistry; RT-PCR, reverse transcriptase quantitative polymerase chain reaction; PD, programmed cell death protein 1; Q61K, (Substitution-Missense, position 61; Q glutamine →K lysine), Q61R (Substitution-Missense, position 61, Q glutamine →arginine R).

metastatic melanomas (10 out 71, 14%), with variable immunoreactivity scores ranging from focal/weak to diffuse/strong. Interestingly, in our series 9 out of 10 ALK positive patients were *NRAS* mutated. Busam and colleagues detected ALK immunoreactivity in metastatic tumors independently on the *BRAF* or *NRAS* mutational status (5).

In melanomas, *NRAS* activating mutations (present in 15-20% of cases) have been associated with aggressive clinical behaviour, and lack of effective treatment options, as well as with poor outcome and lower median overall survival (18,19). In particular, the presence of *NRAS* mutations correlates to shorter survival in stage IV melanomas and it is associated with a higher risk of central nervous system involvement (19). In our study we observed that, among *NRAS*-mutated patients, those ALK positive showed a more favourable outcome in term of DSS, when compared to ALK negative ones. Even though the statistical significance of this observation needs to be confirmed in a larger cohort of patients, its interpretation could open new scenarios. The observation that ALK expression in metastatic melanomas plays a physiological or pathological function still remains an open issue. In our series no ALK translocations or alternative isoforms were detected. This observation suggests that ALK expression is most likely related to the neuroectodermal origin of melanoma cells (20). Indeed, it has been shown that ALK protein is variably expressed in the cytoplasm and/or nucleus of developing central and peripheral nervous system during embryogenesis, and its expression is maintained in the adult at lower level in several tissues, including keratinocytes and melanocytes (http://www.proteinatlas.org/ENSG00000171094-ALK/tissue/skin) (2,13,21,22). The correlation between ALK expression and *NRAS* mutation could be ascribed to the fact that *NRAS* mutations occur at the stage of neural crest and are an early somatic event in the development of the majority of melanomas (22,23). The central nervous system (CNS) is a frequent site of disease progression in melanoma patients, with palliative radiotherapy usually being administered to the CNS metastasis. Recently, a combination of RT and systemic immunotherapy has been proposed for the treatment of stage IV melanoma (24). ALK positive patients could represent a novel subgroup to treat with multimodal therapy comprehensive of TKI, as described in NSCLC brain metastases (25). However, additional clinical studies are needed to determine the efficacy of targeted therapies in melanomas expressing ALK (26).

A larger study is desirable to better clarify, confirm, or possibly rule out the effective ALK reliability as a possible indicator of less aggressive pattern in *NRAS*-mutated metastatic melanoma patients. If confirmed, ALK positive/*NRAS* mutated metastatic melanomas could represent a novel clinical entity and a new therapeutic challenge in metastatic melanoma patients.

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Availability of data and materials

The data that support the findings of the present study are available from Città della Salute e della Scienza Hospital of

Torino, Department of Medical Sciences University of Torino, but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Città della Salute e della Scienza Hospital of Torino and Department of Medical Sciences University of Torino.

Authors' contributions

RP, EM and SOA designed and supervised the study, obtained funding and wrote the manuscript approved by all authors. EP and EB performed the RT-PCR experiments. FL contributed substantially to the design and execution of immunohistochemistry experiments, and preparation of the manuscript. LB and MP provided samples, and designed and supervised the pathological evaluation of samples. SR and MTF supervised the clinical data management and contributed to the statistical analysis.

Ethics approval and consent to participate

The present study was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and within guidelines and regulations by the Research Ethics Committee of the University of Turin. The present study was approved by the Research Ethics Committee of the University of Turin.

Patient consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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