KIT genetic alterations in anorectal melanomas

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ABSTRACT

Background Mucosal melanomas (MM) represent a heterogeneous tumour population that exhibits site-specific molecular profiles.

Aims In a multicentre retrospective study, we investigated *KIT* aberrations in primary anorectal (AR) melanomas compared with melanoma metastatic to the gastrointestinal (GI) tract.

Methods Primary AR MM (n=31) and GI metastatic melanoma (n=27) were studied for *KIT* mutations on exons 11, 13, 17 and 18 by high-resolution melting analysis, direct sequencing and c-KIT expression by immunohistochemistry. Selected cases were also investigated for increased *KIT* gene copy number by fluorescent in situ hybridisation.

Results Functional *KIT* mutations were demonstrated in 11/31 (35.5%) of AR melanomas and in 1/26 (3.8%) of GI melanoma metastases (p=0.004). A significant difference emerged between primary and metastatic MM with regards to KIT-positive immunostaining (p=0.002). Immunohistochemical c-*KIT* protein overexpression did not correlate with *KIT* mutational status. Increased *KIT* copy number was demonstrated in 5/20 AR primary cases.

Conclusions The rate of functional mutations in *KIT* is significantly higher in AR MM than in GI metastatic melanoma. KIT protein overexpression does not correlate with *KIT* mutations and cannot be used for screening purposes. Recognising the molecular heterogeneity of MM helps to identify patients who require a different therapeutic approach.

INTRODUCTION

Melanomas of the gastrointestinal (GI) tract are rare neoplasms, often representing metastatic disease located in the small bowel.[1] In autopsy series, 43.5%–60% of patients who die of cutaneous melanoma have GI metastases.[2–4] Nevertheless, mainly being asymptomatic, <5% of metastases to the GI tract are clinically diagnosed during the patient's lifetime.[1] Primary mucosal melanomas (MM) account for approximately 1.3% of all melanomas.[5] Anorectal (AR) melanomas constitute 23.8% of primary MM, whereas head and neck region, the female genital tract and the urinary tract represent 55.4%, 18% and 2.8% of cases, respectively.[5] Although rarely, MM in the GI tract have been found in the oesophagus, stomach, small and large bowel.[6 7]

GI melanomas are associated with a severe prognosis regardless of the therapy employed.[6] For current and more effective personalised therapies, incorporation of genetic signatures into the morphological classification of melanoma is mandatory.[8 9] In recent years, the use of kinase inhibitors

in patients with melanoma with documented *KIT* mutations has shown promising results.[10–16]

The reported frequency of *KIT* mutations in MM varies between 5.4% and 38% (mean 17.6%; median 17%).[17–32] Frequent association between *KIT* mutations and overexpression of c-KIT protein has been reported,[17 18 20–22 25 26 29] however, data are still conflicting.[15 19 30–33]

To identify the molecular signature of primary AR melanomas, we investigated *KIT* aberrations by evaluating *KIT* mutational status and c-KIT protein immunohistochemical expression in a series of 31 primary AR melanomas compared with 27 cases of cutaneous melanoma metastatic to the GI tract. Selected AR cases were also tested for *KIT* gene copy number abnormalities by fluorescent in situ hybridisation (FISH) analysis.

MATERIALS AND METHODS

Tumour tissue samples

We searched the patient database at the Pathological Anatomy Units belonging to the Tumour Institute of Tuscany (ITT) network (Italy), at the Department of Pathology, National Cancer Institute 'Fondazione G. Pascale' of Naples (Italy) and at the Department of Oncology and Haematology, Papa Giovanni XXIII Hospital of Bergamo (Italy), for cases diagnosed between 1991 and 2013 as primary or metastatic melanoma involving the GI tract. Institutional Review Board was informed and consented in the study. Histological sections were reviewed by two pathologists independently to confirm the diagnosis. In each case, a representative formalin-fixed, paraffin-embedded block was selected and used for immunohistochemistry as well as for molecular studies. Since exons 11, 13, 17 and 18 are described as common mutation sites of *KIT* in melanomas,[17 22] the study focused on the detection of somatic mutations in these exons. FISH analysis was performed in seven cases to exclude the presence of t(12;22)(q13; q12) translocation (r/o soft parts/clear cell sarcoma). FISH was also employed for *KIT* amplification analyses and high-resolution melting analysis (HRMA) and Sanger sequencing were used for mutational testing. For detailed description of immunohistochemistry, FISH and gene mutation analysis, see online supplementary file 1.

Evaluation and statistics

Immunohistochemical stains were evaluated for the percentage of labelled cells and the intensity of immunoreactivity according to the grading criteria suggested by Alexis and coauthors.[34] The amount of immunopositive cells was reported as follows: 0, no staining or weak staining in individual cells; 1+,

spotty weak staining of groups of cells; 2+, diffuse weak staining or moderate staining of up to 50% of cells; 3+, moderate staining of >50% to 75% of cells; 4+, moderate to strong staining of >75% of cells. For statistical purposes, we then divided cases into two groups based on KIT immunohistochemical expression: melanomas overexpressing KIT (scored as 3+ or 4+) and melanomas with low/absent KIT expression (tumours scored as 0/1+/2+).

We accessed the Sanger COSMIC (Catalogue of Somatic Mutations in Cancer) databank (http://www.sanger.ac.uk/genetics/CGP/cosmic/) in order to compare previously reported *KIT* mutations with our results. We have taken into account only *KIT* functional mutations that are known/expected to affect the protein phenotype.

The software SPSS for Windows V.17.0 (SPSS, Chicago, Illinois, USA) was used for statistical analysis. A two-tailed Pearson's χ^2 test was performed, and a value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Patient characteristics and follow-up data

Among patients affected by primary AR melanomas (n=31), age ranged from 56 to 89 years (median 73 years) and included 64.5% (20/31) women and 35.5% (11/31) men. Mean follow-up period was 18.3 months (data were available for 28/31 patients). Anatomical sites of GI metastases (n=27) included small bowel (n=15), large bowel (n=5), stomach (n=3) and oesophagus (n=1). Three patients presented with multiple lesions, comprising different and/or distant GI organs and the omentum. Age ranged from 26 to 82 years (median 63 years) and included 66.7% (18/27) men and 33.3% (9/27) women. Mean follow-up period was 23.5 months (data were available in 21/27 cases).

Histological features of AR melanomas

AR melanomas generally presented as exophytic, diffusely ulcerated lesions. Median tumour size was 3.3 cm (range 0.4–7 cm). Sixteen AR melanomas (16/31; 51.6%) were amelanotic; melanin pigment, at least focally, was present in the remaining cases. Epithelioid melanomas represented the most common subtype (17/31; 54.8%), whereas epithelioid and spindle (n=11; 35.5%) and spindle (n=3; 9.7%) melanomas were less frequently encountered. Primary AR melanomas were all invasive and Breslow thickness ranged from 5 to 15 mm (mean 10.5 mm) with a mitotic index varying from 6 to 30 mitoses/mm^2 (mean 11.9 mitoses/mm^2). Of the nine surgical resection specimens, five were confined to the visceral wall, whereas extension to the perivisceral fat was observed in the remaining cases.

Immunohistochemical results

Overall, c-KIT overexpression (score 3+/4+) was observed in 18/31 (58.1%) primary AR melanomas and in 5/27 (18.5%) metastatic cases. Absent or low c-KIT expression (score 0, 1+, 2+) was demonstrated in 13/31 (41.9%) primary tumours and in 22/27 (81.5%) metastatic cases (figure 1). This difference was statistically significant (p=0.002).

Mutational analysis results

Mutational analysis of the *KIT* gene was performed in all primary AR melanomas and in 26/27 metastatic cases.

In AR melanomas, four cases were found to have mutations on exon 11: the L576P mutation (n=3) and a three-nucleotide deletion p.Y578_D579del (n=1), previously reported in the Sanger COSMIC databank (figure 2). Seven cases showed mutations on exon 17: the p.Y823D mutation (n=6) and the silent p.R804R mutation (n=1). On exon 13, the p.K642R mutation was identified in one case.

Among metastatic melanomas, one of three gastric cases harboured the p.Y823D mutation on exon 17, whereas in a small bowel MM a silent mutation (p.V555V) on exon 11 was detected. No genetic alterations were detected on exon 13.

Exon 18 resulted wild-type in all 57 MM cases.

A statistically significant difference emerged in the frequency of functional *KIT* mutations (missense and frameshift mutations) between primary and metastatic cases (11/31, 35.5% vs 1/26, 3.8%, p=0.004).

Genotype/phenotype correlation

Among the 22 samples with immunohistochemical score 3+ or 4+, 45.5% (10/22) harboured functional mutations, whereas 54.5% (12/22) were *KIT* wild-type. In contrast, among the 35 samples with immunohistochemical score 0/ 1+/ 2+, only 5.7% (2/35) harboured the p.Y823D mutation on exon 17, whereas 33/35 (94.3%) were *KIT* wild-type.

KIT copy number increase

Extra copies of *KIT* were found in 5/20 AR melanomas (25%). These tumours showed c-KIT overexpression, score 3+ (n=1) and 4+ (n=4). At sequencing analysis, four cases were *KIT* wild-type, whereas one also harboured the p.Y823D mutation (figure 3).

DISCUSSION

The most striking result of the present study is that the frequency of functional *KIT* gene mutations is significantly higher in AR MM than melanomas metastatic to the GI tract (35.5% vs 3.8%, p=0.004). Previous studies reported a range of *KIT* mutations in MM of the GI tract between 5.4% and 38%
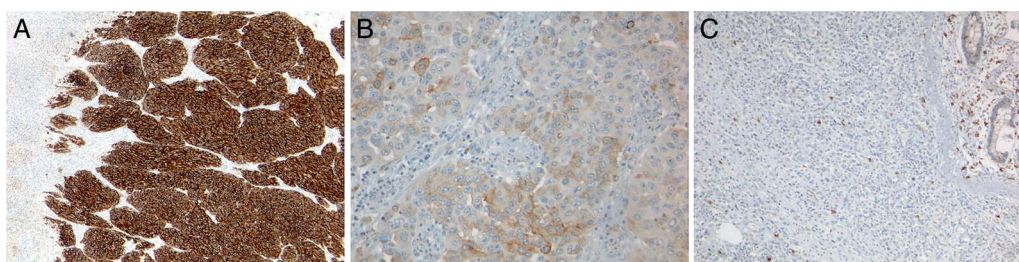

Figure 1 (A) Diffuse, strong immunohistochemical expression of c-KIT in a case of primary anorectal melanoma (original magnification ×10); (B) a case of cutaneous melanoma metastatic to the small bowel showing weak c-KIT immunostaining (original magnification ×20); (C) lack of c-KIT-positive cells in a metastatic melanoma of the small bowel (original magnification ×10).
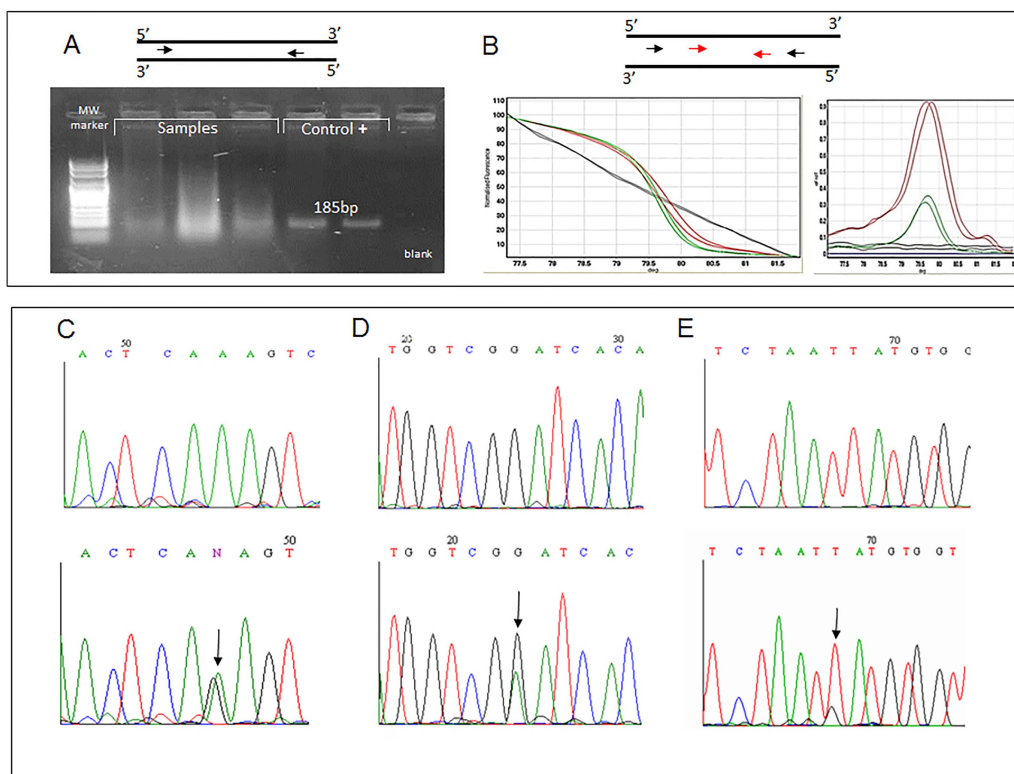
Figure 2 *Upper row*: schematic representation of the preAmp-high-resolution melting analysis (HRMA) methodological approach. (A) Absence of specific amplification on *KIT* exon 17 after a direct PCR; (B) specific amplification on *KIT* exon 17 demonstrated after the preAmp-HRMA protocol, which includes a second amplification with internal primers (tested sample: red line; positive control: green line; direct PCR: black line). *Lower row*: electropherograms of mutated samples. (C) K642R mutation detected in the exon 13, consequently to an AAA>AGA substitution; (D) CGG>CGA substitution responsible of the R804R silent mutation; (E) the p.Y823D mutation caused by a TAT>GAT substitution.

(median 17%).[17–32] Regarding AR MM the reported frequency varied between 0% and 67% (median 15%).[18 20 23 24 26 30 32]

In our series, the frequency of mutated samples was higher than the median frequency previously acknowledged. The wide range of *KIT* frequencies so far reported in AR melanomas may reflect different inclusion criteria and small sample size bias, with number of cases varying between 2 and 40 cases (mean 17.4; median 21).[18 20 23 24 26 30 32]

Differentiating primary from metastatic GI melanomas can be challenging.[6 7] Suggestive features of the primary nature of GI melanoma include lack of concurrent or previous removal of a melanoma or atypical melanotic lesion from the skin, lack of other organ involvement and in situ changes in the overlying or adjacent GI epithelium.[35] Differential diagnosis of MM includes clear cell sarcoma and GIST. Clinical presentation, appropriate

immunohistochemistry and molecular biology tests should be considered to avoid diagnostic misinterpretation with relevant therapeutic implications.

To the best of our knowledge, this is one of the largest series of primary and metastatic GI tract melanomas so far reported.

Among the 11 AR MM, three tumours harboured the L576P mutation on exon 11. These patients were treated with imatinib and are still alive and with response after 10, 12 and 14 months, respectively. In our series, seven patients harboured the p.Y823D mutation, which appears to determine a loop alteration in the c-KIT protein that, in turn, may predict resistance to imatinib in GIST.[36] No similar data have been reported in in vitro or in in vivo melanoma models. Recent data suggest that flumatinib, an inhibitor of BCR-ABL/PDGFR/KIT, has superior efficacy compared with imatinib or sunitinib against GIST cell
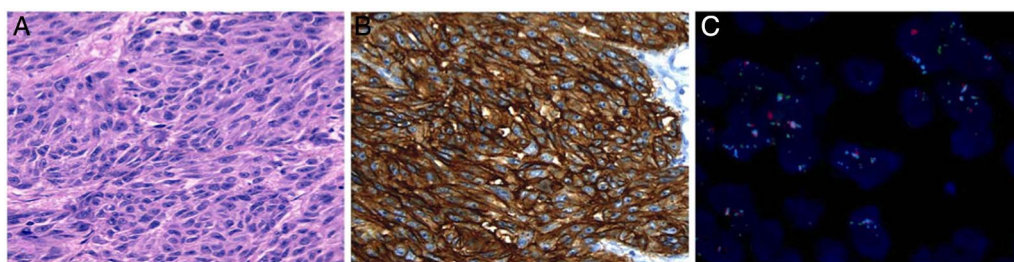

Figure 3 (A) A case of anorectal melanoma resulted wild-type for *KIT* (original magnification ×20); (B) strong and diffuse c-KIT immunohistochemical staining, original magnification 40×; (C) fluorescent in situ hybridisation analysis for *KIT* showing an increase in fused signals for probes to 4q12 (*LSI 4q12 Tri-Colour Rearrangement Probe—Vysis-Abbott Molecular*).

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lines with the secondary mutation p.Y823D.[36] Since KIT inhibitors are target-oriented agents, we could hypothesise that drugs overcoming loop mutations may be an effective therapy in MM with KIT mutations conferring imatinib resistance.[16 37 38]

Overall, 12/45 (26.7%) KIT wild-type tumours showed a high percentage of c-KIT strongly positive cells (scores 3+, 4+) at immunohistochemical analysis. Among these, four cases showed an increased KIT copy number at FISH analysis. One AR melanoma with increased KIT copy number also presented the p.Y823D mutation on exon 17. KIT increased copy number and amplification have been reported in primary MM, either with or without mutations.[17–19 26–28 30 31] It has been suggested that KIT gene amplification is an unusual event in AR melanomas, although modest increase in KIT copy number can be seen in up to one-third of cases.[18 19] Increased KIT copy number, co-overexpression of cyclin-dependent kinase 4 and KIT, epigenetic mechanisms and autocrine/paracrine stimulation of KIT receptor have all been proposed to explain c-KIT protein expression in KIT wild-type cases.[17 22 28 39–41] However, KIT wild-type amplified tumours do not appear to be sensitive to tyrosine kinase inhibitors.[37 42]

Overall, our results suggest that immunohistochemical c-KIT overexpression does not correlate with KIT functional mutation. Although KIT mutations rarely occur in the absence of c-KIT protein expression,[18 22] CD117-negative staining cannot reliably rule out the presence of the mutation.[15 30–33]

In summary, our data suggest that the incidence of activating KIT mutations is higher in AR MM than in melanoma metastasising to the GI tract. KIT-mutated MM are susceptible for therapy with specific kinase inhibitors and the recognition of the molecular heterogeneity of these tumours may help to identify patients requiring a different therapeutic approach.

Take home messages

- ▶ A significant number of anorectal (AR) mucosal melanomas (MM) harbour KIT activating mutations.
- ▶ Immunohistochemical c-KIT overexpression does not correlate with the presence of KIT activating mutations.
- ▶ In AR MM, the spectrum of KIT genetic aberrations is wide and specific KIT mutations may have different sensitivity to c-KIT inhibition.

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