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ANATOMICAL PATHOLOGY

Immunohistochemical CD271 expression correlates with melanoma progress in a case-control study

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Summary

Putative cancer stem cell (CSC) markers have arisen from melanoma mouse and *in vitro* models, but their expression in paraffin embedded patient samples relative to clinical outcome remains largely unexplored. Rather than cells of the tumour bulk, conceivably, CSC drive tumour progression. Accordingly, complete eradication may prevent melanoma relapse. Because elevated tumour-cell proliferation is an established indicator of aggressive disease, this study aimed to investigate the correlation between melanoma recurrence and proliferation of putative CSC that express CD271, CD166, or CD20. Additionally, the expression of these markers was studied in naevi, melanomas, and their recurrence. In melanoma patients, 30 with relapse (cases) and 30 without (controls) were matched for tumour thickness, ulceration, Clark level, subtype, site, gender, and age. One paraffin-embedded section of the patients' primary melanoma (n = 60), relapse (n = 21), and naevus (n = 17) were immunohistochemically double-stained for Ki-67/MART1 and single-stained for CD271, CD166, and CD20. Their whole slide images were aligned as virtual quadruple stains. Image analysis established proliferation indices of each putative stem cell marker and the tumour bulk in addition to the markers' percentage level in tumour areas and the epidermis. In cases vs controls, median dermal proliferation indices (no./mm^2) were 211 vs 103 (p = 0.04) for CD271, 512 vs 227 (p = 0.3) for CD166, 184 vs 97 (p = 0.3) for CD20, and 95 vs 103 (p = 0.6) for the tumour bulk. Of additional interest, epidermal CD271$^+$ keratinocytes totalled 8.8% in naevi and 0.98% in melanomas (p = 0.0007). Even though differences between naevi and melanomas also were observed for CD166 in both the epidermis (p = 0.002) and dermis (p = 0.006), they were visually less apparent. CD20$^+$MART1$^+$ cells were absent in half of the melanomas, and all naevi and relapses. In conclusion, high levels of CD271$^+$Ki-67$^+$MART1$^+$ cells were linked to melanoma relapse as opposed to common Ki-67 indices in this particular case-control study. With further investigation, such cells could be potential targets of therapy. Especially, loss of epidermal CD271$^+$ keratinocytes seemed necessary for melanoma development; hence, identification may serve as a diagnostic tool with additional research.

Key words: Cancer stem cells; CD271; CD166; CD20; immunohistochemistry; Ki-67; melanoma; metastasis; naevus.

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INTRODUCTION

High metastatic potential and resistance to adjuvant therapy characterises cutaneous melanoma, although novel immune checkpoint inhibitors and genetically targeted agents recently have demonstrated a survival benefit in patient subgroups.[1] Correspondingly, 10-year survival rates of 70% for regional metastases and 15% for distant metastases have recently been reported. For localised melanoma, this rate varied from 75% to 98% according to the most important prognostic markers: tumour thickness, ulceration, and proliferation.[2,3] Surprisingly, few thin melanomas with low proliferation indices also metastasised; some even decades after surgical resection.[2]

A possible existence of melanoma stem cells has recently been suggested from human-to-mouse xenograft studies.[4,5] Rather than cells of the tumour bulk, they are thought to drive tumour progression. Their possible ability to enter a relatively quiescent state and tolerate chemotherapy is suggestive of melanomas' unyielding ability to reoccur.[6]

The classic hypothesis states that melanoma arises from a mature, differentiated melanocyte that has undergone a series of genetic alterations. Dependent on their mutational status and distinct signals from the tumour microenvironment, all tumour cells have the same potential for differentiation and proliferation.[4,7,8] In contrast, cancer stem cells (CSC) are, presumably, capable of asymmetrical cell division (two sister cells with different fates are produced, which is detectable by differences in size, morphology, gene expression pattern, or the number of subsequent cell divisions);[9] hence, one identical CSC may be re-produced alongside a sibling with only a terminal ability to differentiate and proliferate.[6,10,11] While some evidence points at the existence of a CSC model,[4,5] some studies have also found that the majority of tumour cells may be able to form new melanomas.[12,13] Both tumour models could exist or co-exist within tumours and vary amongst melanoma subtypes.[7]

Nevertheless, existence of cancer-cell populations with stem-like properties has been proposed for cancers of the breast, brain, colon, and skin.[4] Especially CD271, CD166, CD20, CD133, and ABCB5 have been suggested as potential markers of stemness in melanoma;[4,5,14,15] but so far, few groups have studied the markers in routine formalin fixed, paraffin embedded samples from melanoma patients in relation to clinical outcome. Some connection between their dermal immunohistochemical presence and disease outcome has been demonstrated.[16,17] Yet, stem cells reside naturally in both melanomas and naevi, and potential

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immunohistochemical markers may be co-expressed in other cell types, e.g., fibroblasts and lymphocytes.[18] Thus, specific recognition within melanocytic cells seems necessary.

With preservation of morphology and skin architecture, simultaneous analysis of putative CSC markers relative to, e.g., a melanocytic marker (such as MART1) seems feasible by virtual immunohistochemical multiple stains (whole-slide images of serial stains are digitally superimposed to form one image). Yet, to ensure accurate cell-to-cell alignment, only a limited number of slides can be combined (three to four). In this study, the three immunohistochemical markers CD166, CD271, and CD20 were of particular interest because: (1) seemingly, CD166 remains the only putative CSC marker that has been recognised as an independent prognostic marker in a fairly large clinical study of melanoma patients with time-to-event data (n = 107);[16] (2) $CD271^+$ cells seemingly play a very important role in both physiological and pathological states of the skin, although their involvement in tumour formation remains controversial;[19] and (3) anti-CD20 is currently the only therapy that may aim putative CSC cells.[20]

CD166, also termed activated leucocyte cell-adhesion molecule (ALCAM), is a transmembrane glycoprotein involved in cell adhesion and cytoskeletal anchoring. It is presumably present on the surface of mesenchymal stem cells, but also on activated T cells and monocytes, epithelial cells, neurons, and fibroblasts.[18]

CD271, also known as p75NTR, is a transmembrane receptor for the family of nerve growth factors called neurotrophins. The receptor has been associated with cells of neural crest origin from which melanocytes are derived.[21] In sensory and sympathetic neurons, CD271 promotes cell death, and a similar role outside the nervous system seems evident, however, this remains to be fully clarified.[19]

CD20 is usually expressed on B lymphocytes, but expression has also been observed on small subsets of melanoma cells.[20,22] In fact, elimination of only $CD20^+$ melanoma cells in a study of mice eradicated tumours to the same extent as elimination of all cancer cells,[22] which indicates stem-like properties. In addition, adjuvant anti-CD20 therapy has shown promising results in a small patient cohort (n = 9) with advanced melanoma.[20]

Moreover, the ability of cells to sustain proliferative activity is a hallmark of cancer.[23] But even though the proliferation index of haematoxylin and eosin (H&E) and Ki-67 stains are renowned markers of prognosis in many malignancies including melanoma,[2,24] current therapeutics that target the proliferative tumour bulk fail.[4,6] Although CSC often are thought relatively quiescent with low proliferative rates,[4,25] their specific proliferation index may still indicate a tumour's potential for propagation. Yet, the relation between CSC proliferation and disease outcome remains largely unexplored. In a study of glioblastomas, the immunohistochemical presence (±) of $Ki\text{-}67^+CD133^+$ cells was a strong independent marker of both overall and progression-free survival.[26] In one fairly small study of childhood melanoma, $Ki\text{-}67^+CD133^+$ cells were only detected in the primary tumours of patients with metastases.[27]

This study aimed to investigate the correlation between recurrent melanoma and the proliferative potential of putative CSC that express CD271, CD166, or CD20 in patients with cutaneous melanoma, and to study the expression of these markers in naevi, melanomas, and melanoma relapses.

MATERIALS AND METHODS

Specimens

In 60 patients with cutaneous melanoma, 30 cases with recurrent disease were matched with 30 control subjects without a pathological record of melanoma relapse. A formalin fixed, paraffin embedded tissue block was collected from each primary tumour, the recurrence of cases (n = 21), and, if possible, a successive naevus of the patient (n = 17).

Relevant clinical and pathological characteristics were recorded for all melanoma patients with an excision biopsy diagnosed at the Department of Pathology at Aarhus University Hospital or Randers Regional Hospital, Denmark, between 1992 and 1996; an area prior to the sentinel lymph node technique and the mitotic count. An attempt to match cases and controls for primarily Breslow thickness and ulceration, but also Clark level of invasion, tumour subtype and site, gender, and age resulted in 30 pairs. Still, some pairs differed in ulcerative status (n = 2), with one Clark level (n = 2), between the subtypes superficial spreading and nodular (n = 5), in location (n = 11), or gender (n = 9). The median thickness for all patients included was 1.65 mm, and the mean difference between cases and controls was −0.013 mm (95% CI, −0.19 to 0.17 mm; p = 0.88). The median age at diagnosis was 51 years, and the mean difference between cases and controls was 6 years (95% CI, −7 months to 12 years; p = 0.074). Table 1 displays characteristics of all patients.

Because fine-needle aspiration biopsies were excluded (nine distant metastases), 21 tissue blocks from the relapse of cases were included from various pathology departments in Denmark (six distant metastases, 13 regional lymph-node metastases, and two local skin recurrences).

Of all patients, 24 had a subsequent naevus removed. Only the 17 lesions that were diagnosed in Aarhus were included in the study (one junctional, eight dermal, five compound, and three dysplastic compound naevi). If the patient had a history of numerous naevus removals, close excision dates of the naevus and melanoma were prioritised. The median time between their excisions was 14 months (range 3 days–23 years).

Using newly made H&E stains, experienced dermatopathologists confirmed all initial diagnoses and features for matching cases and controls.

Median follow-up times for patients still alive were 21 years (range 19–24 years) for primary melanomas and 19 years for naevi (range 11 months–24 years). Pathology reports were the primary source of follow-up, but medical journals were consulted if necessary. For control subjects, 25 were still alive without evidence of metastatic disease; five died after >20

Table 1 Patient and tumour characteristics

Feature	No. (%)
Ulceration	
Absent	52 (87)
Present	8 (13)
Clark level of invasion	
II	4 (7)
III	15 (25)
IV	40 (67)
V	1 (2)
Histopathological subtype	
Superficial spreading	43 (72)
Nodular	15 (25)
Lentigo maligna melanoma	2 (3)
AJCC 7th edition T subcategory	
T1 (Breslow thickness ≤1.00 mm)	15 (25)
T2 (Breslow thickness 1.01–2.00 mm)	21 (35)
T3 (Breslow thickness 2.01–4.00 mm)	20 (33)
T4 (Breslow thickness >4.00 mm)	4 (7)
Site of primary tumour	
Trunk	18 (30)
Head	3 (5)
Extremity	39 (65)
Gender	
Female	43 (72)
Male	17 (28)

AJCC, American Joint Committee on Cancer; T, primary tumour.

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