CLINICAL REPORT

Malignant Melanoma Arising in Patients with a Large Congenital Melanocytic Naevus: Retrospective Study of 10 Cases with Cytogenetic Analysis

Caroline LACOSTE[1], Marie-Françoise AVRIL[2], Annonciade FRASSATI-BIAGGI[1], Nicolas DUPIN[2], Bertrand CHRÉTIEN-MARQUET[3], Emmanuel MAHÉ[4], Christine BODEMER[5], Béatrice VERGIER[6], Arnaud DE LA FOUCHARDIÈRE[7] and Sylvie FRAITAG[1]

[1]Departments of Pathology and [3]Plastic Surgery, Necker-Enfants Malades Hospital, APHP, Paris Descartes – Sorbonne Paris Cité University, Institut Imagine, [2]Department of Dermatology, Cochin Hospital, APHP, Paris-Descartes-Sorbonne University, Paris, [4]Department of Dermatology, Argenteuil Hospital, Argenteuil, [5]Department of Dermatology, Necker-Enfants Malades Hospital, National Reference Centre for Genodermatoses (MAGEC), Paris Descartes – Sorbonne Paris Cité University, Institut Imagine, Paris, [6]Department of Pathology (Sud), Haut-Lévêque Hospital, CHU Bordeaux, Bordeaux University, PESSAC, Bordeaux, and [7]Department of Pathology, Leon Bérard Center, Lyon, France

Large congenital melanocytic naevi (LCMN) represent the main risk factor for development of melanoma in childhood. This retrospective study of 10 cases of melanoma in patients with LCMN used fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH) (6 cases) to elucidate the clinical, histological, and cytogenetic characteristics of this rare disorder. Six melanomas were found within the LCMN, the others in lymph nodes, subcutis and brain. The LCMN was located on the trunk in 8 cases, with satellite naevi in 6 cases. Two distinct groups emerged: 5 melanomas that developed before the age of 10 years and the other after 20 years. The mortality rate was 60% and clearly correlated with clinical stage at diagnosis. Histological diagnosis was difficult in only 2 patients in whom neither immunohistochemistry nor FISH were helpful. Otherwise, CGH showed a high number of chromosomal aberrations leading to a formal diagnosis. *Key words: melanoma; giant congenital naevus; fluorescence* in situ *hybridization; comparative genomic hybridization.*

Malignant melanoma (MM) is rare in children, representing only 1–3% of paediatric tumours (1). One of the main risk factor for development of melanoma in children is congenital melanocytic naevus (CMN), especially large CMNs (LCMN) (2) measuring more than 19.9 cm at their widest point (3). The risk of occurrence of LCMN is only 1/20,000 births (4). Approximately 130 cases of MMs associated with LCMN have been published so far, including only 40 cases with histological data (5–20). Most of these are isolated observations but a few series with a short follow-up are also available (5–8). The epidemiological, clinical, and histological characteristics described in these cases appear to differ from those of sporadic MMs and from MMs developed on small congenital naevi (2, 21, 22). The risk of MM occurring in combination with a LCMN is approximately 2%, and is proportionate to the size of the naevus (23). The histological diagnosis of such MM can be difficult because LCMN themselves sometimes present clinical melanoma simulators and display certain atypical and worrisome histological features, including proliferative nodules (PNs). This phenomenon explains why melanomas may have been over-diagnosed, with a resulting overestimation of the risk. There is little molecular data on melanoma tumourigenesis available in the literature. However, we do know that LCMN have a specific molecular signature with somatic mutations in the *NRAS* gene, while acquired or small congenital naevi mainly present BRAF mutations (22).

With a view to improving the characterization of these melanomas, we performed a retrospective study involving 10 patients and, for some of them, performed fluorescence *in situ* hybridization (FISH) and/or comparative genomic hybridization (CGH) studies.

MATERIALS AND METHODS

We conducted a retrospective study over a 20-year period. Patients were recruited in the medical departments of the same university. The inclusion criterion was: MM established by histopathology in patients with a LCMN, located in the skin either within or outside the LCMN or in another part of the body.

Methods

Clinical, histological and immunohistochemical data were collected. Clinical data on the congenital naevi were: sex, size, location, presence or absence of satellite naevi, date of the first excision of the CMN, and whether excision appeared complete. Slides were reviewed: the size of the naevus samples was recorded and the surgical margins, especially their depth, were examined. Clinical data collected for cutaneous and extracutaneous MM included age at diagnosis of MM, location from which the melanoma sample was obtained, clinical stage at diagnosis, outcome, number of metastases, deaths, survival time after diagnosis and treatment. All MM were subjected to histological review by two dermatopathologists (SF, CL), one of whom is specialized in paediatric melanocytic lesions (SF).

The study was approved by the ethics committee.

Histopathology and immunohistochemical studies

Studies were carried out on 3-μm-thick sections of formalin-fixed, paraffin-embedded tissue using standard techniques. The histological data collected for MM were the exact location in the skin, dermis and/or subcutis, shape and size of the cells (epithelioid, lymphoblast-like, naevoid, fusiform or others), the extent of anisocytosis and/or anisonucleosis, presence and number of mitotic figures and presence of necrosis and inflammation. The number of mitoses was counted by mm^2. The technique of immunostaining was performed using the Bond Max (Leica, New Castle, UK) according to the manufacturer's recommendations. The primary antibodies used were Ki67 (dilution 1/100, pH6, DAKO), HMB45 (dilution 1/150, pH6, DAKO, Glostrup, Denmark) and p16 (dilution 1/2, pH 9, Cintec, Roche; Mannheim, Germany). For Ki67, the percentage of labelled nuclei was assessed by counting 10 large fields at a magnification of ×40. When HMB45 was used, the percentage of labelled cells was quantified, and homogeneous or heterogeneous features were noted. In the case of p16, the percentage of labelled cells was noted. Labelling was considered positive when a cell was positive in the cytoplasm and/or the nucleus.

Molecular fluorescence in situ *hybridization study*

This was performed in 7 patients using the 4-colour Vysis Melanoma FISH Probe kit, used according to the manufacturer's recommendations (Abbott France, Rungis). FISH analysis was performed by 2 trained cytogenetics specialists (MC, EL). Criteria for FISH positivity were those published by Gerami et al. (24). In parallel, 3 probes located on 3 different chromosomes (8q22 (ETO), 21q22 (AML1) and CEP17) were used to study numerical chromosomal aberrations on the same slides. For 2 patients who lost p16 expression on immunostaining, an additional FISH on the 9p21 locus was performed.

Comparative genomic hybridization study

CGH study was carried out after sample selection and DNA extraction in 6 patients. To evaluate the percentage of tumour cells, 4-μm sections were cut from formalin-fixed paraffin embedded (FFPE) tumour blocks and stained using standard HES. Small amounts of tumour were then collected by scraping 6–10 unstained slides containing 4-μm FFPE sections. DNA extraction was performed using the QIAamp DNA micro kit (Qiagen #56304, Sussex, UK) and DNA was eluted in 20 μl of DNAse-free water. DNA concentrations were measured using a Qubit® 2.0 fluorometer, and the quality of the DNA was assessed by electrophoresis on the Bioanalyzer 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA). For CGH, fragmentation and labelling were performed according to the manufacturer's recommendations (Agilent Technologies, Santa Clara, CA, USA), with some modifications related to FFPE material. Co-hybridization was performed on 4×180K AgilentSurePrint G3 Human whole-genome oligonucleotide arrays (Agilent #G4449A). Slides were washed, dried and scanned on the Agilent Surescan scanner according to the manufacturer's recommendations. Scan images were processed using Agilent Feature Extraction softwareV11.0 and the analysis was carried out using the Agilent Genomic Workbench software V7.0.

RESULTS

Clinical data (Table SI[1])

Ten 10 patients were enrolled in the study (5 females, 5 males). All patients had a large size CMN. The LMCN consisted of 5 torso naevi, 3 bathing-trunk naevi, 1 on the face and 1 on the left upper limb. Multiple satellite naevi were observed in 6 cases. Eight patients underwent repeated surgical procedures of LCMN in order to remove the naevus completely, which was achieved in only 8 patients. However, even if it seemed complete clinically, the excision was always incomplete histologically at the deep margin of the naevus, with meanage at first surgical excision under 2 years. MRI was not performed systematically at birth or in the first months of life, except for patient 2.

The mean age at the time of MM discovery was 19 years, with extremes of 1 year and 57 years. Five occurred before 10 years of age and 5 after 20 years of age. In 5 patients the primary MM was located in cutaneous sites and presented as a nodule, which was either polypoid or deeply located in the subcutis. For the 5 remaining patients, lesions were located either in a superficial axillary or inguinal regional lymph node, or in the central nervous system (2 patients). At diagnosis, the disease was classified as stage II in patients 1, 2, 8 and 10, stage III in patients 4 and 5, and stage IV in patients 3, 6, 7 and 9. For patients 6 and 7, neurological symptoms led to the discovery of a solitary brain melanoma on the brain computed tomography (CT) scan, which was excised and pathologically analysed. In both patients, a short-term relapse occurred with melanomatous meningitis in one case and multiple brain metastases in the other case.

The first treatment was a large excision of melanoma whatever the clinical stage, except for 1 case (patient 6), who was treated only with chemotherapy. Stage III patients were treated with radical lymph node dissection in addition to large excision of primary melanoma. Therapy for stage IV patients consisted of chemotherapy and radiotherapy in one case.

For the 5 remaining patients, initial lesions were located in a superficial inguinal lymph node, on the central nervous system or the sphenoid area and adjacent soft tissues. At diagnosis, the disease was classified as stage II in patients 1, 2, 8 and 10, stage III in patients 4 and 5, and stage IV in patients 3, 6, 7 and 9. Adjuvant therapy for stage III and IV consisted of chemotherapy and/or radiotherapy.

The mean follow-up period in the series was 6.6 years. At the time of the study, 4 patients aged 19, 27, 33 and 67 years were alive and 6 patients had died of MM. The mean age of death was 17.3 years. Mean survival after the diagnosis was 6.6 years, with a minimum of 3 months and a maximum of 26 years.

Histopathological and immunohistochemical data (Table SII[1], 10 cases)

All the MMs developing within the LCMN consisted in a nodule located either in the dermis or in both dermis and subcutis without affecting the epidermis. They were composed of large or medium-to-large epithelioid, lymphoblastic or pleomorphic cells. Anisocytosis and anisokaryosis were moderate to marked. Mitotic activity varied from weak (≤5 mitoses/mm²) to brisk (≥10 mitoses/mm²), and was unrelated to the primary or metastatic nature of the lesions. Necrosis and inflammation was present in cases 5 and 10, respectively (Figs S1A, B[1], S2A–C[1] and S3A[1]).

Immunohistochemistry showed that the proliferation index Ki67 ranged from 5% to 40% and was closely correlated with the mitotic index in all but one case. No correlation was found between mitotic figures and/or a low proliferation index and outcome or metastatic stage. HMB45 staining was mainly cytoplasmic and diffuse. It was negative in one case. P16 staining showed cytoplasmic and/or nuclear positivity in 40–100% of cells, but was negative for the 3 cases arising in adults (Figs S1C–E[1]; S2D–F[1]; S3B–D[1]).

Molecular data, FISH and CGH (Table SIII[1])

FISH analysis of childhood MM showed generalized polysomy extrapolated from polysomy of chromosomes 6, 11, 8 and 21 with no structural abnormalities usually found in classic melanoma. With regard to the patients whose MM developed in adulthood, this study showed generalized polysomy, trisomy of chromosome 1 and 6 and structural abnormalities with RREB1 gain and MYB deletion. Only 2 patients out of 3 with negative p16 expression on immunostaining (patients 8 and 9) were explored by FISH because the third had Bouin tissue fixation. These 2 patients did not present with 9p21 locus deletion (CDKN2A gene). The adjacent naevi were always controlled in our 6 patients and no structural or numerical chromosomal abnormalities were ever observed (Fig. S1F[1]).

CGH array profiles (patients 1, 3, 4, 6, 7, 8) for each patient tested showed a great number of imbalances, often with a combination of gains and losses not limited to whole chromosomes (international standardized formula in Table SIII[1], Figs S1G[1], S2G[1] and S3E[1]). Most profiles showed one or more recurrent chromosomal abnormalities already identified in melanomas occurring in large congenital naevi.

DISCUSSION

MM combined with a CMN is a rare entity (23). According to 3 major reviews of the literature, the risk of developing MM in patients with a LCMN is now estimated to be approximately 2% (23).

We report here 10 cases of MM associated with LCMN. All these MM arose in patients with large or even giant CMN, most after clinically complete surgical removal of the original lesion. The size and location of the CMN and its association with multiple satellites naevi seem to influence the risk of occurrence of a MM. The majority of studies report that MM arise on CMN located on the trunk in 90–100% of cases (25–27), measuring over 40 cm in diameter in 75% of cases and the naevus was associated with satellite naevi in 80–100% of cases (23, 25, 27, 28), which was the case in 6 of our 10 patients. In 2 patients MM occurred in the brain and in 2 others, within a naevus located outside the trunk, such as on the face or arm. In the literature MM was located in the skin in 50–100% of cases (23, 25, 27–29) both within and outside the CMN (27), similar to our 6 cutaneous cases, and was extracutaneous in 8% and 7% in 2 recent series (23, 29). Metastatic melanomas can also be diagnosed without any identified primary melanoma, as was the case in patient 4.

Despite almost complete removal of LCMN in 8 of our patients, surgery failed to prevent the development of MM, and the role played by surgical excision of LCMN remains controversial. Some authors advocate that the risk of malignant transformation may be reduced by surgery, whilst others consider that surgical excision activates melanocytes, in some cases prompting the development of a malignant clone (30).

As is the case in other published studies (25, 27), the sex ratio in our series was 1, but other studies report a female bias (26, 31). Two distinct trends emerged: early melanoma before the age of 10 years and late melanoma after 20 years of age. This result is similar to the findings published in the literature reporting a mean age at MM diagnosis varying from 12.6 to 15.5 years, and including a similar period of early occurrence (<10 years) in 25–70% of cases contrasting with a second period of late occurrence (>20 years) in 27–50% of cases (2, 23, 25, 27–29). It is important to emphasize the possibility of late-onset MM in patients with a LMCN. Our results suggest that patients should be properly informed about this risk of late-onset MM and that lifelong follow-up may be appropriate.

The 60% mortality rate in this series is similar to previous publications reporting from 35.2% to 77.5%. However, the mean age of death at 17.3 years is somewhat older. We observed only 20% of deaths before 10 years, compared with 25–90% of cases according to published studies (25, 27). In our series, the median survival after diagnosis of MM was 6.6 years, with a survival time of less than one year in 50% of cases.

The course of the disease was mostly related to the stage at diagnosis. Among stage II patients, 2 are alive 7 and 10 years after MM excision, respectively, one patient had regional lymph node relapses treated by radical lymph node dissection and is alive 10 years after

excision of the primary MM, whereas the last patient developed brain metastasis and died 11 years after MM diagnosis. Among stage III patients, one patient is alive 26 years after regional lymph node dissection, whereas the second patient died of disseminated disease 3 months after regional lymph node excision. All stage IV patients at diagnosis died of the disease within a short period of time (less than 6 months).

The gold standard in diagnosis of MM is histopathology (32). However, so far, only approximately 40 cases have been reported with histological analysis (5–20). In our series skin lesions consisted in tumoural nodule located in the dermis and/or the subcutis, always separated from the epidermis by a grenz-zone. These features were similar to those reported in the literature. In the majority of described cases skin lesions consisted in tumoural nodules located in the dermis and/or the subcutis composed of epithelioid large cells, showing marked nuclear pleomorphism, large nuclei with nucleoli and areas of necrosis and/or inflammation and numerous mitoses, as in our series, except numerous mitosis observed in only 3 cases of ours series. Diagnosis of MM was quite easy from the histopathological features alone in 8 cases, but more difficult in patients 2 and 3. In these patients, the progression to malignancy appeared to have been gradual, with a shift from a benign or ambiguous pattern to more worrisome features. For these ambiguous cases immunohistochemistry contributed little to the diagnosis of malignancy. In compliance with the literature (33), the proliferation index Ki67 in our series varied from 5% to 40%, but remained low in our 2 ambiguous cases that died from MM, and was not predictive of outcome in all cases including metastatic outcome. A low index is therefore not a reliable means of ruling out malignancy and a high index can be useful, but not for neonates, in whom numerous mitotic figures and/or a high proliferation index are common (34, 35, 36).

In 9 out of our 10 patients, HMB45 expression was diffuse and cytoplasmic in 40–95% of naevus cells; including our 2 ambiguous cases and negative in the remaining patient (patient 10). HMB45 expression is therefore not helpful for the diagnosis of malignancy.

In our study, p16 was expressed in 7 MM cases and was absent in 3 cases. Strikingly, these 3 patients with negative p16 were adults, with ages ranging from 27 to 57 years. The p16INK4 is a cyclin-dependent kinase receptor acting as negative regulator of cyclin D-dependant kinase and is a critical gate keeper at the G1-S check point. Thus, gradual loss of p16INK4a expression correlates with the advancing stages of melanocytic progression. The expression of p16 by childhood MM vs its absence in MM developed in adulthood may be due to the limited role played by solar radiation in paediatric melanomas.

Since the combination of histopathological analysis and immunohistochemistry was not always sufficiently specific to confirm MM, FISH and CGH studies were also performed with a view to assessing the potential usefulness of these technique in accessing malignancy and to compare the abnormal CGH profiles with similar published data (37).

FISH study revealed chromosomal abnormalities in MMs, but not in the adjacent naevi. The abnormalities differed depending on whether the MM had developed during childhood, with only numerical type similar to those described by the same team in proliferative nodules located within LCMN (results to be published), or during adulthood, with numerical and structural type. However, if this study seems to predict different pathways for childhood and adulthood melanoma, it was non-contributory for malignancy in ambiguous cases.

The first CGH data were reported in 2002 by Bastian et al. (38) for 5 MMs developing within LCMN in patients aged from 18 months to 65 years. In all 5 cases, they reported structural abnormalities similar to the alterations observed in adult-type sporadic melanoma. In our study, the CGH results obtained in 6 cases confirmed its usefulness for the diagnosis of malignancy in ambiguous situations. The level of complexity of these profiles was typical of a malignant tumoural process, especially when compared with the flat profiles reported in benign cases and proliferative nodules (34, 35). However, on such a small sample of cases that could still represent a heterogeneous group of tumours in genetic terms, it is still difficult to identify one or more recurrent chromosomal abnormalities specific of melanomas occurring in large congenital naevi. The most frequent anomalies were gain of 1q (3/6), loss of 5q (4/6), gain of 6p (3/6), gain of 8 (4/6), loss of 9p (4/6), loss of 10q (3/6), loss of 14 (3/6), loss of 16q (3/6), loss of 17p (3/6) and loss of 18 (3/6), although no specific combination of these anomalies was observed. As described by Bastian et al. (38), we did not find a significant difference in our cases from sporadic melanomas occurring in adults. Thus, in ambiguous cases in which IHC and FISH were of little help, CGH seems to be a useful tool establishing a complex profile that allows reliable diagnosis of malignancy and guides the clinician towards an immediate appropriate treatment.

Conclusion

Malignant melanomas associated with LCMN are rare and therefore poorly understood. Our data showed that MMs occurred mostly with LCMN located on the back and trunk and in the skin. It developed at different ages, with 2 specific periods: in childhood before 10 years of age and in adulthood after 20 years of age, despite prophylactic surgical removal of the LCMN. Outcomes were linked to the clinical stage at diagnosis, but no histological prognostic factors were identified. CGH seems to be the most useful tool to establish diagnosis of malignancy if histological findings are not straightforward.

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