Identification of circulating malignant cells and its correlation with prognostic factors and treatment in uveal melanoma. A prospective longitudinal study

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Abstract

Purpose Most uveal melanoma patients (UMP) do not show evidence of metastases upon diagnosis. However, despite local tumour control, ~50% of them will develop metastases. These findings suggest that malignant cells may have already disseminated by the time of initial diagnosis. The purpose of the study was to detect circulating malignant cells (CMCs) in UMP and to correlate them with prognostic factors and therapy.

Methods Nested reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect CMCs. In each UMP, blood was collected every 3 months. In each visit, 20 RT-PCR tests were performed. The date of diagnosis, largest tumour dimension, type, and date of treatment were obtained.

Results A total of 30 UMP were enrolled. Five patients were enrolled at the time of diagnosis and 25 patients between 1 and 17 years following diagnosis. No UMP showed clinical evidence of metastasis. A total of 136 visits were registered, 1360 samples collected, and 2720 RT-PCRs performed. CMCs were identified in 29 patients in 119 visits (87.5%). However, in each visit, a low number of positive tests were recorded. CMCs were found in newly diagnosed, irradiated, enucleated, and observed patients regardless of tumour size and time period following treatment.

Conclusions Uveal melanoma (UM) is not a localized ocular disease. CMCs were recorded

at initial diagnosis confirming the early metastatic nature of UM. CMCs were present following treatment, including enucleation, demonstrating that CMCs are capable of disseminating and surviving, possibly as micrometastasis, which would contribute to the pool of CMCs at a later stage. Systemic therapy should be evaluated.

Eye (2007) 21, 752–759; doi:10.1038/sj.eye.6702322; published online 31 March 2006

Keywords: uveal melanoma; metastatic uveal melanoma; circulating malignant cells; micrometastasis; RT-PCR; minimal residual disease

Introduction

Uveal melanoma (UM) is a relatively rare malignancy. It is most commonly seen in Caucasian patients with a median age at presentation of 55 years.¹ New treatment modalities such as radiation therapy have replaced, in many instances, the more conventional approach of enucleation, significantly decreasing patient morbidity. Mortality, however, has not been affected.² Forty to fifty per cent of all patients will still die of metastatic disease³ within 9–10 years following diagnosis, despite early diagnosis of the primary tumour, successful local treatment, and close follow-up for the detection of metastasis. Life expectancy upon diagnosis of metastasis is extremely poor owing to the limited success of systemic therapy.⁴

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Received: 8 June 2005
Accepted in revised form: 29 January 2006
Published online: 31 March 2006

Part of this work was previously presented as a poster at ARVO 2005

According to the current standard of care, patients are screened for asymptomatic metastatic disease⁵ upon diagnosis and annually/biannually using liver function tests (LFTs) and imaging studies (chest X-rays, liver ultrasound, MRI).⁶ Recently, however, the Collaborative Ocular Melanoma Study Group reported that the use of LFTs followed by diagnostic imaging studies carry a very low sensitivity for the detection of metastatic disease.⁷ Proper assessment of the extent of the disease is therefore one of the major challenges in the current management of uveal melanoma patients (UMP). Therefore, the development of a more sensitive test for an accurate staging of these patients is critical, as it will select appropriate candidates for earlier systemic therapy.

Several studies performed on patients with breast,⁸ prostate,⁹ cancer, and cutaneous melanoma¹⁰ have concentrated on the idea of analysing peripheral blood samples to detect circulating malignant cells (CMCs) using reverse transcriptase-polymerase chain reaction (RT-PCR). Results from these studies are promising, as the technique was found to be sensitive and specific, and results are associated with clinical stage and prognosis.^{11–14} Furthermore, the presence of CMCs has been found to be a useful predictor of relapse and death.¹⁵ In patients with cutaneous melanoma, Tyrosinase and MART-1/Melan A (melanocyte-specific genes) have been successfully used to identify CMCs in peripheral blood samples. The technique relied on the fact that melanocytes are not normally present in the blood and therefore, the detection of the expression of a melanocyte-specific gene in blood is a suitable indicator of the presence of circulating melanoma cells.¹⁶ The analysis of peripheral blood samples is particularly appealing in UM, as the tumour is remarkable for purely haematogenous dissemination.

The purpose of this study was to detect CMCs in the peripheral blood of a cohort of UMP and to correlate their presence with tumour size, modality of treatment, and time period between treatment and CMCs detection. In addition, changes in the CMC's profile were evaluated during a longitudinal follow-up.

Patients, materials and methods

We conducted a prospective clinical study at the MUHC site, Royal Victoria Hospital, McGill University, Montreal, Canada. The study received approval from the Clinical Research Ethic Review Board. All UMP attending the Oncology Clinic were eligible to participate. During the initial visit, a complete ocular examination was performed, which included ultrasound measurement of the tumour size. Chart review was carried out to obtain the date of diagnosis of the UM, the largest tumour dimension by ultrasound, and, if

applicable, the modality and date of treatment. The time period between treatment and enrolment (T T/E) was calculated.

The first blood sample was obtained at enrolment and every 3 months thereafter. In each visit, 30 ml of peripheral blood in EDTA anticoagulant was obtained. To avoid possible contamination with skin melanocytes, the first 10 ml collected was stored for future studies. The remaining 20 ml was subdivided into 10 aliquots of 2 ml each. Each aliquot was tested for the presence of CMCs using Melan A and Tyrosinase as markers by nested RT-PCR (one visit = 20 nested RT-PCR tests = 10 Melan A and 10 Tyrosinase tests). If one or more of the 20 nested RT-PCR test was positive, the patient was considered positive for that visit. Finally, the presence of CMCs was correlated with tumour location, tumour size at diagnosis, tumour size at enrolment, treatment, and T T/E.

Each step of the technique was carefully selected according to the results of extensive sensitivity and specificity assays (data not shown). These assays were performed using positive controls consisting of increasing concentrations of spiked human UM cells in peripheral blood of healthy volunteers. The minimum detection level was found to be 10 melanoma cells in 2 ml of blood. The technique tested negative for both markers at all times when analysing blood from non-spiked controls ($n=30$) and non-melanoma patients ($n=30$), which included patients with small choroidal nevi, uveitis, patients enucleated for blind painful eye, trauma, or non-melanocytic tumours.

The identification of CMC in peripheral blood was accomplished through the following steps: (1) isolation of leucocytes and CMCs from whole peripheral blood using Ficoll-Paque Plus; (2) extraction of RNA from leucocytes and CMC using Trizol reagent; (3) DNase treatment to eliminate genomic DNA; (4) reverse transcription of mRNA to cDNA; (5) primary amplification with melanoma markers by PCR; and (6) secondary amplification with melanoma markers by nested PCR.

Briefly, 30 ml of fresh whole blood was obtained by peripheral venepuncture. To avoid possible contamination with skin melanocytes, the first 10 ml collected was separately processed to extract serum and/or plasma and was stored for future studies. The remaining 20 ml of EDTA-treated blood was subdivided into 10 aliquots of 2 ml each and processed within 30 min. Each aliquot was individually processed to obtain the mononuclear layer using Ficoll-Paque Plus method (Amersham Biosciences; catalogue # 17-1440-02) in accordance with the manufacturer's instructions. Diluted whole blood was carefully layered on top of 3 ml of Ficoll-Paque Plus and centrifuged for 35 min at 1350 r.p.m. at 18–20°C. The lymphocyte layer was

isolated and the pellet was saved at -80°C for RT-PCR. As part of the longitudinal study, all samples from the same patient collected during multiple visits were kept at -80°C until the end of the trial when they were simultaneously run in an effort to decrease day-to-day laboratory variability.

Total RNA was extracted by the one-step guanidinium thiocyanate method.¹⁷ The concentration and purity of the RNA were determined spectrophotometrically by absorption at 260 and 280 nm using an Ultrospec 2000 spectrophotometer (Biochrom Ltd, Cambridge, UK).

Two micrograms of total RNA was incubated with 1 U/ μl DNase (Deoxyribonuclease I, amplification grade; Invitrogen) to eliminate genomic DNA contamination and then the DNase was inactivated by addition of EDTA and heat. The above-mentioned mixture (11 μl) was immediately reverse transcribed by adding 10 μl of a reaction mixture containing 500 mg/ml oligonucleotides 12–18 (Amersham Biosciences), 10 mM dNTP mix (Invitrogen), $5 \times$ first strand RT buffer (Invitrogen), 0.1 M DTT (Invitrogen), 40 U/ μl RNA guard (Amersham Bioscience) and 200 U reverse transcriptase from Moloney murine leukemia virus (Invitrogen). An RT-negative control was performed for each specimen of RNA. After incubation for 50 min at 37°C , heating at 70°C for 15 min inactivated the reaction. The reaction mixture was diluted five-fold (100 μl) and used for PCR amplification or stored at -20°C .

PCR was performed using 5 μl of cDNA (from RT dilution) and 9.7 μl of a reaction mixture containing 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 50 mM MgCl_2 , 10 mM dNTP mix (Invitrogen), 10 μM of each primer (Tyrosinase: sense 5' TTGGCAGATTGTCTGTAGCC and antisense 5' AGGCATTGTGCATGCTGCTT; Melan A: sense 5' CACTCTTACACCACGGCTGA and antisense 5' TAAGGTGGTGGTGAAGTCTTG) (Sheldon Biotechnology Centre, McGill University, Canada), and 1 U *Taq* DNA polymerase (Invitrogen) in a reaction volume of 50 μl . The sample in which the cDNA was omitted served as a PCR negative control. The amplification of cDNA sequences was performed using a PTC-100 programmable Thermal Controller (MJ Research Inc., Massachusetts, USA). After the initial denaturation at 94°C for 4 min, the PCR cycling protocol consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for Tyrosinase and at 54° for Melan A for 45 s, and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. The amplification products of Tyrosinase (284 bp) and Melan A (264) were visualized by electrophoresis using ethidium bromide staining in a 1.8% agarose gel (Invitrogen).

Nested RT-PCR was performed using 2 μl of the previous product with the addition of the same PCR reaction mix and the addition of different primers that

bound to sites within the primary amplimers (Nested RT-PCR primers: Tyrosinase sense 5' GTCTTTATGCAA TGGAACGC and antisense 5' GCTATCCCAGTAAGT GGACT and Melan A sense 5' TCATCGGCTGTTGGTA TTGTAG and antisense 5' ATAAGCAGGTGGAGCA TTGG) (Sheldon Biotechnology Centre, McGill University, Canada). The sample in which the primary PCR product was omitted served as nested PCR-negative control. Additional 25 cycles were performed with an annealing temperature of 56°C for Tyrosinase and 55°C for Melan A to obtain a final product of 207 bp for Tyrosinase and 245 bp for Melan A. The final product was visualized similarly to those obtained in the previous step.

Strict measurements were employed to reduce RT-PCR false positive results and contamination. Negative controls for every RT-PCR step and one negative sample were processed with each run and remained negative throughout the entire experiment. Each PCR run also contained two positive controls spiked with 10^6 and 10 UM cells. To evaluate RNA quality, 50 samples were randomly selected and checked for the expression of the housekeeping gene, B actin (125 bp) and GAPDH (246 bp).

Results

A total of 30 UMP were enrolled in the study and followed for a maximum of 21 months. The number of visits per patient range from 1 to 7, with 21 patients (70%) complying with a minimum of four or more visits. During the entire study, 136 visits were recorded with 1360 blood samples collected and 2720 nested RT-PCR tests performed, 1360 for Tyrosinase and 1360 for Melan A.

Sixteen patients were women and 14 patients were men with a median age of 66 years at diagnosis of the primary tumour and 71.5 years at enrolment. Twenty-two patients were irradiated, four were surgically treated (two enucleations, two iridocyclectomies), and four were observed without treatment. Two irradiated patients were secondarily enucleated before enrolment, one owing to tumour re-growth and one owing to neovascular glaucoma.

Five of 30 patients were enrolled in the study at the time of initial tumour diagnosis. Blood samples were collected before and after treatment (three irradiated and one enucleated) in four of these patients. The remaining 25 cases were enrolled after treatment, with an average time period between diagnosis and enrolment of 3.5 years and between treatment and enrolment of 2.4 years. Seven patients were enrolled within 1 year following diagnosis of the primary tumour, 17 patients between

1 and 6 years of diagnosis, and six patients between 7 and 17 years following diagnosis.

Regarding tumour size at initial diagnosis, four patients had small size melanoma (<10 mm basal diameter or 3 mm in height), 17 medium (11–15 mm basal diameter or 3.1–8 mm in height), six large tumours (>15 mm in basal diameter or >8 mm in height), and three unknown. Tumour size at study enrolment was distributed as follows: 11 patients had small size melanoma, 13 medium, and one large. In the remaining five patients, the tumour was surgically excised before enrolment. None of the 30 patients showed clinical evidence of metastatic disease during the length of the study.

In 29 of 30 UMP, we identified the presence of CMCs. CMCs were found in 87.5% (119 of 136) of visits registered throughout the study. In each visit, however, an average of one of 10 nested RT-PCR tests for Tyrosinase and two of 10 tests for Melan A were recorded as positive. Furthermore, over the course of the entire study, only 20.4% (278 of 1360) of all nested RT-PCR for Melan A and 8.3% (113 of 1360) of all Tyrosinase tests collected were positive.

Based on the size of the tumour at the time of initial diagnosis, patients were divided into four groups: small, medium, large, and unknown. All four groups have comparable profiles of CMCs with similar numbers of positive and negative visits registered throughout the study. (Figure 1a–c) In the majority of positive visits ($n=105$ of 119, 88.2%), a low number of positive tests were recorded per visit (less than four of 10 tests for either Melan A or Tyrosinase). In the remaining visits ($n=14$; 11.8%), a higher numbers of positive tests were recorded (five or more test for either marker). A total of 18 negative visits for both markers were seen. During the longitudinal follow-up, the three categories of visits (negative, weakly positive, and strongly positive) were intermixed with no apparent profile in all four groups.

The impact of therapy on the presence of CMCs was analysed in four patients, who were enrolled in the study at the time of initial primary tumour diagnosis. In these patients, pre- and post-treatment samples were obtained (Figure 2 and 3). Three patients were irradiated and one enucleated. All four patients remained positive for the detection of CMCs following treatment. In the group of 25 patients enrolled following primary tumour treatment, five had surgical excision of the primary tumour 1–7 years before enrolment. In all five patients, CMCs were identified over the course of the study (Figure 2).

When analysing the impact of using two markers, Melan A and Tyrosinase, to increase detection of CMC, we found that from a total of 119 positive visits, 56 visits (47.1%) were only positive for Melan A, nine visits (7.6%)

were positive only for Tyrosinase, and in 54 visits (45.3%) coexpression of both markers was found.

No correlation was found between the presence of CMCs and the size of the primary tumour at diagnosis, the size of primary tumour at enrolment, the type of treatment, and the time period between treatment and enrolment.

Discussion

Traditionally, metastatic disease was interpreted as a random process that occurred during late stages of tumorigenesis.¹⁸ Limited research was carried out in the field, as it was believed that there were no general rules that could govern metastasis development.

Only recently, metastasis has evolved into a subject of extensive investigation that has enlightened and challenged our traditional view on the subject. New insights have clearly demonstrated that metastasis is neither a random process nor a late manifestation of a growing tumour.¹⁹ It has been recognized that several metastasis-related genes act in delicate concert, as a toolbox, to predetermine the tumour metastatic signature.²⁰ Also, there is increasing evidence for early tumour cell dissemination and early metastatic disease that evolves in parallel with the primary tumour.²¹

Primary tumours and clinically detectable metastatic disease can be seen as progressive specific 'time points' within the evolution of the disease. Between these two points, there is a continuum of disease progression that includes the initiation of systemic disease. The exact 'time point' of initiation of systemic disease remains to be elucidated, as current screening techniques are known to underestimate its occurrence. Interestingly, in our study, the presence of CMCs was recorded as early as at the time of initial diagnosis despite the size of the primary tumour. Based on this observation, we can estimate that the 'time point' of initiation of systemic disease in UMP has taken place early during tumorigenesis confirming that UM is an early metastatic tumour. This result concurs with and complements limited studies performed in the field. Eskelin *et al*²² and Singh²³ estimated that the time of micrometastasis in UM is 2.9 years earlier than the diagnosis of the primary tumour, indicating that dissemination has occurred before conservative treatment of the primary tumour.

Our understanding of the steps that follow CMCs dissemination is remarkably poor. We were able to detect CMCs not only at the time of initial primary tumour diagnosis but also many months to years later. In accordance with our view of cancer as a clonal proliferation of cells, the primary tumour must be the first source of CMCs during the early stages of tumour cell dissemination (primary tumour cell dissemination).

Figure 1 Presence of CMCs in three UMP with small (a), four patients with medium (b), and six patients with large (c) size tumours at the time of diagnosis. The longitudinal follow-up showed comparable profile of CMCs between the three groups with a similar number of positive and negative visits. Although a low number of CMCs were most commonly observed, these cells may randomly increase, decrease, or even briefly disappear in individual patients. Only representative patients are displayed in the graph. Profiles showed are based on Melan A results.

We may speculate that the primary tumour could continue to be a source of CMCs over time and this may explain the presence of CMC in irradiated patients.

Nevertheless, CMCs were also detected many months to years following resection of the primary tumour (Figure 2). These patients tested positive when CMCs

Figure 2 Longitudinal follow-up of UMP with surgically excised tumours. Tumours were excised up to 7 years before the first blood collection. All patients showed the presence of circulating malignant cells in most visits. The arrow indicates the time of enucleation for patient 23 (-●-). Profiles showed are based on Melan A results. IC: iridocyclotomy; E: enucleation; RX: radiotherapy.

Figure 3 Effect of treatment on the presence of CMCs. These four uveal melanoma patients were treated during the study. Three patients (-■- small size tumour, -▲- small size tumour, -●- medium size tumour) were irradiated and one patient (-X-, large size tumour) was enucleated. The arrows indicate the time of treatment for each patient. All four patients continued to test positive for CMCs at least once after treatment. Profiles showed are based on Melan A results.

originated from the primary tumour were expected to have cleared from peripheral blood.²⁴ They did not have at any time another clinical evident source of CMCs such as local tumour recurrence or clinically detectable metastatic disease. These observations point to at least another potential source of CMCs. We can hypothesize that disseminated cells do have the capability of target organ colonization possibly as single cells or micrometastasis. They may remain occult for long

periods of time, maybe years, in a state known as tumour cell dormancy.²⁵ These micrometastatic foci may be capable of generating CMCs that will recirculate at a later phase (secondary tumour cell dissemination). Of note, whether UM seeding affects exclusively the liver or is widespread remains speculative.

In this context, it is mandatory to introduce the concept of minimal residual disease (MRD) to better describe this subclinical state. MRD is characterized by the presence of

tumour cells despite the successful removal of the primary tumour, and despite the patient being judged to be free of cancer by standard clinical method.²⁶ The presence of MRD may explain the lack of improvement on mortality rate over several decades despite early and successful local treatment. It may also help to understand the similar outcome reported in patients treated with enucleation or radiotherapy.²⁷

Interestingly, only 40–50% of patients will progress to develop clinically evident metastatic disease within 9–10 years. The discrepancy between the presence of CMCs in all of our patients and the expected outcome may be explained by the well-known phenomenon of metastatic inefficiency.²⁸ The nature of the metastatic cascade implies that all the steps in metastasis formation should be completed in order to develop clinically detectable metastatic disease. Based on this study, we demonstrated that tumour cells are capable of disseminating and have the potential for developing secondary colonization. However, the final metastatic outcome could be truncated by the presence of rate-limiting steps that may, for instance, negatively regulate growth expansion at the target organ, as has been demonstrated in other cancers.²⁹ Genetic,³⁰ immunological, and environmental factors,³¹ among others, may influence the final outcome.

Few studies investigating the presence of CMCs in UMP have been published to date. Tobal *et al*³² and Smith *et al*³³ were among the first to use RT-PCR to detect tyrosinase-positive CMCs in peripheral blood in UMP. Although their preliminary work on a small group of patients was promising, they failed to detect CMCs in a larger group of patients.³⁴ Surprisingly, in our study, most of our patients tested positive for the presence of CMCs. The discordance between these results and ours may be explained by technical differences in sample collection and processing, including a larger amount of blood analysed in our study (20 *vs* 3.2 ml), a higher number of nested RT-PCR performed per visit (20 *vs* 1), and the addition of a second RT-PCR marker, Melan A, which in our hands proved to be more sensitive than Tyrosinase. The rationality of our approach was to help reduce false-negative results, as it is speculated that there is a low number of CMCs in peripheral blood at a given point of time. This speculation may be correct, as only a low number of tests performed per visit were positive. This low pattern of positivity was maintained during the longitudinal follow-up in the majority of patients (Figure 1a–c).³⁵

A limitation of this type of study is the assumption that the cells encountered in the circulation are in fact malignant. Cells identified by RT-PCR should express specific melanocytic markers exclusively found in both benign skin melanocytes and melanoma cells. Technical measurements can be taken to avoid potential

contamination of the sample during skin venepuncture (see Patients, materials and methods). Also, benign melanocytes do not circulate in blood, so it is assumed that positive results are owing to the presence of disseminated melanoma cells. This is the assumption that is made in all reports published in the field of skin melanoma in which the presence of CMC was investigated. Furthermore, CMCs are also identified in different types of tumour, such as colon, prostate, or breast, which have no benign equivalents in the skin. We are actively working in a new technique that will allow us to isolate intact CMC from patients' blood and subject them to gene chip analysis to prove their malignant nature. We had accomplished this goal in our UM animal model in which CMCs were isolated in a reduced number of animals.

At this point, we do not fully understand the short- or long-term significance of detecting CMCs in the peripheral blood of UMP, other than the potential for metastasis. It is, however, possible to conclude that at the time of initial diagnosis, the first few steps of the metastatic cascade, intravasation and dissemination, have already taken place regardless of the size of the primary tumour. Patients remain in a state of MRD that is unaffected by the local tumour treatment. Whether these patients will progress to develop clinically detectable metastatic disease will depend on factor/s that exerts its influence on any of the subsequent steps of the metastatic cascade. It is, therefore, of utmost importance to consider the existence of MRD when planning therapy in UMP in order to achieve a long-term therapeutic effect. Appropriate therapy that targets disease at an asymptomatic stage and prevents metastasis formation should be beneficial.

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