The role of c-kit and imatinib mesylate in uveal melanoma

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Abstract

**Background:** Uveal melanoma (UM) is the most common primary intraocular tumor in adults, leading to metastasis in 40% of the cases and ultimately to death in 10 years, despite local and/or systemic treatment. The c-kit protein (CD117) is a membrane-bound tyrosine kinase receptor and its overexpression has been observed in several neoplasms. Imatinib mesylate is a FDA approved compound that inhibits tyrosine kinase receptors, as well as c-kit. Imatinib mesylate controls tumor growth in up to 85% of advanced gastrointestinal stromal tumors, a neoplasia resistant to conventional therapy.

**Methods:** Fifty-five specimens of primary UM selected from the archives of the Ocular Pathology Laboratory, McGill University, Montreal, Canada, were immunostained for c-kit. All cells displaying distinct immunoreactivity were considered positive. Four human UM cell lines and 1 human uveal transformed melanocyte cell line were tested for in vitro proliferation assays (TOX-6) and invasion assay with imatinib mesylate (concentration of 10 µM).

**Results:** The c-kit expression was positive in 78.2% of the UM. There was a statistical significant decrease in the proliferation and invasion rates of all 5 cell lines.

**Conclusion:** The majority of UM expressed c-kit, and imatinib mesylate does decrease the proliferation and invasion rates of human UM cell lines. These results justify the need for a clinical trial to investigate in vivo the response of UM to imatinib mesylate.

Background

Uveal melanoma (UM) is the most common primary intraocular tumour in adults [1], with an incidence of five to six individuals per million people [2]. Forty percent of UM patients will progress from local to systemic disease developing metastasis that will ultimately lead to death after 10 years of diagnosis, despite the treatment options...
such as local radiotherapy, enucleation and systemic chemotherapy [3].

C-kit (Kit, CD117, stem cell factor receptor) is a 145 kDa transmembrane tyrosine kinase protein that acts as a type-III receptor. The c-kit proto-oncogene, located on chromosome 4q11-21, encodes the c-kit, which ligand is the stem cell factor (SCF, steel factor, kit ligand, mast cell growth factor) [4,5]. Tyrosine phosphorylation by protein tyrosine kinases is of particular importance in cellular signaling and can mediate signals for major cellular processes, such as proliferation, differentiation, apoptosis, attachment, and migration. The role of c-kit expression has been studied in hematologic and solid tumors, such as acute leukemias [6], and gastrointestinal stromal tumors (GIST) [7].

The clinical importance of c-kit expression in malignant tumors relies on the existence of a compound (imatinib mesylate, STI571, Gleevec®, Novartis Pharma AG Basel, Switzerland) that specifically inhibits tyrosine kinase receptors [8]. Moreover, a clinically relevant breakthrough has been the finding of remarkable anti-tumor effects of this compound in GIST, a group of tumors regarded as being generally resistant to conventional chemotherapy [9]. Imatinib mesylate has been approved by the United States-FDA to treat c-kit positive GIST and Philadelphia-chromosome-positive chronic myelogenous leukemia [10]. The purpose of this article is to study the immunexpression of c-kit in UM, as well as the in vitro effects of imatinib mesylate on UM cell lines.

Materials and methods

**Paraffin blocks**

Formalin-fixed, paraffin-embedded blocks from enucleation of primary choroidal melanoma were collected from the archives of the Henry C. Witelson Ocular Pathology Laboratory, McGill University, Montreal, Canada from the years 1980–2004. All the cases have sufficient tumor material for analysis. The tumors with irradiation were excluded.

**Immunohistochemistry**

Immunohistochemistry was performed using the polyclonal anti-CD117 antibody A4502 (Dako, Mississauga, Ontario, Canada). The antibody was applied at a dilution of 1:300 for 18 h at 4°C, after 15 minutes in 10 nmol/l citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase was blocked using 0.3% hydrogen peroxidase diluted in methanol for 30 minutes. A standard avidin-biotin complex (ABC) technique using dianaminobenzidine was used for visualization, with a red colouring stain to avoid misinterpretation in pigmented tumors. A case of KIT-positive Gastrointestinal Stromal Tumor (GIST) was used as control. Negative control sections were incubated with normal rabbit serum instead of the primary antibody.

After tissue processing, all cells displaying distinct immunoreactivity were considered positive, irrespective of staining intensity. We assigned the results of c-kit staining as negative when no staining was present, low when less than 50%, and high when more than 50% of melanoma cells were positive. The immunoreactivity was categorized as cytoplasmatic or membranous expression using the grade system described above. In order to better characterize the c-kit expression in uveal melanomas, the expression in the different cell types (spindle and epithelioid – modified Callender’s classification) was analysed, in the
mixed cell, predominant epithelioid and predominant spindle tumors.

**Cell Culture**

Four human UM cell lines (92.1, SP6.5, MKT-BR, OCM-1) and one human uveal transformed melanocyte cell line (UW-1) were incubated at 37°C in a humidified 5% CO₂-enriched atmosphere. The cells were cultured in RPMI-1640 medium (Invitrogen, Burlington, Ontario, Canada), supplemented with 5% heat inactivated fetal bovine serum (FBS), 1% fungizone, and 1% penicillin-streptomycin purchased from Invitrogen (Burlington, Ontario, Canada). Cells were cultured as a monolayer in 25 cm² flasks (Fisher, Whitby, Ontario, Canada) and observed twice weekly, at every media change, for normal growth by phase contrast microscopy. The cultures were grown to confluence and passage by treatment with 0.05% trypsin in EDTA (Fisher) at 37°C and washed in 7 ml RPMI-1640 media before being centrifuged at 120 g for 10 minutes to form a pellet. Cells were then suspended in 1 ml of medium and counted using the Trypan Blue dye exclusion test.

The UM cell lines 92.1, SP6.5, and MKT-BR were established by Dr. Jager (University Hospital Leiden, The Netherlands), Dr. Pelletier (Laval University, Quebec, Canada), Dr. Belkhou (CJF INSERM, France), respectively. Dr. Albert (University of Wisconsin-Madison, USA) established the OCM-1 and UW-1 cell lines [11,12].

**In Vitro Invasion Assay**

A modified Boyden chamber consisting of a polyethylene teraphthalate membrane (PET) with 8-um diameter pores, precoated with Matrigel, an artificial basement membrane, (Beckton Dickenson Labware, Bedford, MA) was used as previously described [13], to assay for invasive ability. PET membrane without Matrigel was used as a control.

Briefly, 1.25 × 10⁵ cells were added to the upper chamber in RPMI-1640 medium with 0.1% FBS. RPMI-1640 medium with 10% FBS was added to the lower chamber as a chemoattractant to obtain the baseline invasive ability of the cell lines. The effect of imatinib mesylate on invasion was assayed by adding 10 µM of imatinib mesylate to the RPMI-1640 medium supplemented with 10% FBS in the upper chamber. The concentrations of imatinib mesylate was chosen based on the blood levels reported in previous studies with the maximum tolerated dose of imatinib mesylate on clinical trials [14]. The chambers were then incubated at 37°C in a 5% CO₂-enriched atmosphere for 48 hours to allow for cellular invasion through the Matrigel.

|                  | High expression | Low expression | Negative |
|------------------|-----------------|----------------|----------|
| Spindle cells    | 36.8%           | 57.9%          | 5.3%     |
| Epithelioid cells| 41.5%           | 53.6%          | 4.9%     |
| Cell membrane    | 100%            | 30.4%          | ------   |
| Cytoplasmatic    | 100%            | 100%           | ------   |

Table 1: High, low and negative expression of c-kit in different cell types and different patterns of stain.
Non-invading cells were removed from the upper chamber by gently wiping the surface of the membrane with a moist cotton swab. Membranes were removed and then stained using a Diff-Quick staining set, which stains cell nuclei purple and cytoplasm pink. Stained cells were counted microscopically in 20 high-powered fields, randomly. Only cells whose nuclei had completely invaded through the membrane were counted. Each experimental condition, including control, was performed in triplicate and the average number of invading cells was then calculated for all experimental conditions.

Percent invasion was determined for each cell line under each experimental condition using the following formula:

\[
\% \text{ invasion} = \left( \frac{\text{mean number of cells invading through the matrigel}}{\text{mean number of cells migrating through control PET membrane}} \right) \times 100
\]

The cell lines were then ranked according to their invasive ability.

**In Vitro Proliferation Assay**

The Sulforhodamine-B based assay kit (TOX-6, Sigma-Aldrich) was performed as per the National Cancer Institute protocol [15]. Briefly, five human UM cell lines were seeded into wells at a concentration of \(2.5 \times 10^3\) cells per well, in a minimum of six wells per cell line. A row of 8 wells exposed to only RPMI-1640 medium was used as a control. Twenty-four hours following seeding, imatinib mesylate was added to the experimental wells. The concentration of imatinib mesylate was 10 \(\mu\)M [14]. Cells were allowed to incubate for 48 hours following cell seeding. Following this 48 hour period, cells were fixed to the bottom of the wells using a solution of 50% Trichloroacetic acid (TCA) for 1 hour at 4°C. Plates were then rinsed with distilled water, to remove TCA and medium, and air dried. The Sulforhodamine-B dye was added to each well and allowed to stain for 25 minutes. The Sulforhodamine-B dye was subsequently removed by washing with a 10% acetic acid solution and once more allowed to air dry. The dye that was incorporated into the fixed cells at the bottom of the wells was solubilized in a 10 mM solution of Tris. The absorbance of the solute was measured using a microplate reader at a wavelength of 510 nm. This gave a comparison of control cell proliferation rate over 48 hours compared to proliferation rate of cells exposed to imatinib mesylate during the same time period at a dose of 10 \(\mu\)M.

**Statistical Analysis**

The differences in invasion rates under three experimental conditions for each uveal melanoma cell line were determined using the ANOVA test. A p value of less than 0.05 was considered statistically significant. Calculations were computer-based (SPSS 11.5, SPSS Inc., Chicago, Illinois, USA).
Results
Fifty-five cases of UM were studied. Eight seven percent of the tumors (n = 48) were classified as mixed cell type (spindle and epithelioid cells), 9% (n = 5) had predominance of epithelioid cells, and 3.6% (n = 2) of spindle cells.

Seventy-eight percent of cases (n = 43) were found to be c-kit positive (Figure 1A). Among the positive cases, 46.5% (n = 20) presented with what was considered as high expression. All lesions with high immunoreactivity (n = 20) had cytoplasmic and cell membrane expression. Meanwhile, among lesions with low immunoreactivity (n = 23), 100% presented a cytoplasmatic reaction and just 30.4% (n = 7) presented with a cell membrane stain-pattern (Figure 1B). (Table 1)

The percent invasion of cell lines according to the baseline invasion without imatinib mesylate was: MKT-BR (38.4%) > OCM-1 (21.7%) > 92.1 (14.4%) > UW-1 (12%) > SP6.5 (3%). The addition of imatinib mesylate decreased the invasion in all cell lines: MKT-BR (1.03%); OCM-1 (0.1%); 92.1 (0.2%); UW-1 (0%); SP6.5 (0%). The results are shown in Figure 3.

No visible changes in cytomorphology were seen in reaction to the presence of imatinib mesylate (figure 2).

Statistically significant differences between the invasion rates for the control group and imatinib mesylate group were found in all cell lines (T test p value < 0.05).

Discussion
It is known that protein tyrosine kinases (PTK) have an important role in cellular mechanisms, such as differentiation, proliferation and regulatory mechanisms, as well as in signal transduction. C-kit is one of these PTK, which is expressed in a wide variety of human malignancies [16] including chronic and acute myelogenous leukemia [6], GIST [7], mastocytosis [17], small cell lung carcinoma [18], chromophobe renal cell carcinoma [19], cutaneous [20] and UM [16]. As c-kit is expressed in normal interstitial cells of Cajal, the progenitor cell of GIST [7], the present article studies the expression of c-kit in uveal melanomas, as normal choroidal melanocytes do express this marker [21].

We demonstrated that more than 75% of UM from our series are positive for c-kit. This finding, per se, supports the idea of a clinical trial of imatinib mesylate for UM, especially in metastatic cases. Once metastatic disease is detected, no effective method of systemic therapy has been identified [3]. Moreover, not 100% of GIST is positive for c-kit. In fact, 6% of GIST are c-kit negative [22]. Before the imatinib mesylate “era”, metastatic GIST had a median survival times ranging between 10–20 months [23]. Nowadays, imatinib mesylate controls tumor growth in up to 85% of advanced GIST [24], with 90% of acceptable toxicity [25].

In cutaneous melanoma, c-kit is strongly expressed in radial growth phase, and weak or no expression is seen in vertical growth phase and metastatic disease [26]. Therefore, in cutaneous melanomas c-kit expression appears to be related with stage of the disease. To further investigate a similar expression of the c-kit in UM, we observed the cell type (spindle and epithelioid) in which the c-kit was expressed, as it is well known that spindle cell type is less aggressive than epithelioid type [1]. None of the previous studies recorded the cell type in which the expression was occurring. Mouriaux et al [21] compared the c-kit expression with cell type tumor (Callendar’s classification) as a correlation to prognostic factor, but did

| Cell Line | Average Number of Cells in Control PET | Percent Invasion of Cells in Response to 10% FBS | Percent Invasion of Cells in Response to Imatinib mesylate |
|-----------|----------------------------------------|-----------------------------------------------|----------------------------------------------------------|
| MKT-BR    | 247                                    | 38.43% ± 8.5                                  | 1.03% ± 0.2                                               |
| OCM-1     | 246.67                                 | 21.7% ± 1.9                                   | 0.1% ± 0.2                                                |
| 92.1      | 223                                    | 14.4% ± 2.7                                   | 0.2% ± 0.2                                                |
| UW-1      | 66.33                                  | 12% ± 4.8                                     | 0% ± 0                                                    |
| SP6.5     | 266                                    | 3% ± 1.5                                      | 0% ± 0                                                    |
that c-kit would interfere in UM proliferation could be
decrease UM cells proliferation rates [8]. The mechanism
demonstrate that the decrease of proliferation of UM cells
growth factor (PDGF) receptor [30]. We could
tyrosine kinases such as Bcr-Abl and platelet-derived
mesylate selectively inhibits not only c-kit, but also other
exons 2, 8, 9, 11, 13 and 17 [29]. However, imatinib
[28]. Choroidal melanoma does not have alterations of
Cajal, harbouring mutation of c-kit. Mutations are
cerning important differences between cytoplasmatic and
expression is diffuse strong cytoplasmatic and up to 50%
high c-kit expression showed both cytoplasmatic and cell-
membrane pattern of stain. In GISTs, the c-kit
expression is diffuse strong cytoplasmatic and up to 50%
of the cases show cytoplasmatic dot-like (so-called "golgi
pattern") staining [7]. Moreover, there are no studies
concerning important differences between cytoplasmatic
and membranous staining in GIST. Therefore, we hypothesize
that all cases expressing c-kit, cytoplasmatic or membra-
ous, should be considered c-kit positive.

GIST is a sarcoma arising from the interstitial cells of
Cajal, harbouring mutation of c-kit. Mutations are
detected in approximately 71% of tumors, the majority
(over 60%) involving exon 11, and less exons 9 and 13
[28]. Choroidal melanoma does not have alterations of
exons 2, 8, 9, 11, 13 and 17 [29]. However, imatinib
mesylate selectively inhibits not only c-kit, but also other
tyrosine kinases such as Bcr-Abl and platelet-derived
growth factor (PDGF) receptor [30]. We could
demonstrate that the decrease of proliferation of UM cells
with imatinib mesylate was very significant, in the 4 UM
cell lines tested and in the human uveal transformed
melanocyte cell line, compared to the control group.
Other studies also support that imatinib mesylate can
decrease UM cells proliferation rates [8]. The mechanism
that c-kit would interfere in UM proliferation could be
other than c-kit mutation, but further studies are neces-
sary to investigate this hypothesis.

The concentration of imatinib mesylate used for the in
vitro studies was 10 µM. This concentration is equivalent
to the highest drug concentration achieved in the blood of
patients receiving 1000 mg/day of imatinib mesylate, the
maximum tolerated dose reported by clinical trials [14].
At that dose, the blood concentration of imatinib
mesylate ranged from 6 to 10 µM [14]. According to clin-
cial trials, the current treatment for GIST is 800 mg/day of
imatinib mesylate [25]. All-Ericsson et al [16] demon-
strated that concentrations of 10 µM of imatinib mesylate
could inhibit the proliferation of 5 UM cell lines in 50%
(2 of them, OCM-1 and 92.1, were also studied in this
article). The different concentrations of imatinib mesylate
tested had different responses according to the cell line.
Pache et al [29] also had the same conclusion. Moreover,
the last demonstrated that imatinib mesylate does not
influence the proliferation of normal uveal melanocytes.
The human uveal transformed melanocyte cell line UW-1
studied in the present article demonstrated a significant
decrease in proliferation and invasion rates when treated
with imatinib mesylate. UW-1 was originally derived from
uveal melanocytes, and transformed into malignant
melanoma cells throughout culture. We hypothesize that
imatinib mesylate could act in a more general pathway
than c-kit receptor, as it does inhibit UW-1 proliferation
and invasion rates.

The effect on invasion of UM cells in response to imatinib
mesylate has never been published before. Tumor cells
must possess invasive abilities in order for metastasis to
occur. Due to the lack of lymphatics in the eye, uveal
melanoma cells must leave the primary tumor via hema-
togenous dissemination, with metastasis almost exclu-
sively occurring in the liver [2]. Our study demonstrated
that imatinib mesylate markedly reduced the invasiveness
of all cell lines tested. The invasion assay is important to
show the ability of cells to invade a basement membrane,
simulating the escape of cells from the primary tumor, as
well as the implantation of cells at the site of the meta-
stasis. The use of artificial basement membrane can study
the invasive response of cells to drugs by counting the amount
of cells that invade the matrigel layer. A drug that can
inhibit or reduce the invasiveness ability of the UM cells
could be beneficial, as most of the UM are nowadays
treated conservatively [31]. Decreasing the invasiveness
of the tumoral cells, the drug would also decrease the ability
of implantation of cells at the site of metastasis. Therefore,
imatinib mesylate would be beneficial not only for UM
patients that already developed metastasis, but also for
patients without any sign of metastatic disease.
Conclusion
We could demonstrate that primary choroidal melanomas express c-kit and imatinib mesylate decrease the proliferation rate and invasiveness of uveal melanoma cells in vitro. Therefore, our data supports a clinical trial for studying imatinib mesylate in uveal melanoma.

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