Research Article

The effects of cystatin C construct clones on B16F10 in vitro cell behavior

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Abstract

Background: Metastasis is the cause of most cancer related morbidity. A naturally occurring cysteine protease inhibitor, cystatin C, has been reported to inhibit tumor cell metastasis for several different cancer types, however, the mechanism is still unknown. Our study focused on determining which region of cystatin C is responsible for anti-metastatic action by characterizing specific constructs of cystatin C in melanoma cells. In one construct, the N-terminal peptide amino acids 1-10, required for cysteine protease inhibition, were deleted. In the second construct the conserved motif QLVA was altered to GGGG. **Methods and Results:** Net proliferation, migration and invasion of the cystatin C constructs were assessed. The modified Boyden chamber revealed 75% reduced invasion of N-truncated clones compared to control B16F10, similar to cystatin C overexpression. A scratch migration assay also showed a three-fold reduction in migration rate. The QLVA sequence was found to be required for inhibition of B16F10 invasion and migration. Net cell proliferation remained constant between clones. **Conclusions:** Overexpression of cystatin C with or without cysteine protease inhibitor activity inhibits the migration and invasion, but not proliferation, of B16F10 melanoma cells. The conserved cystatin sequence, QLVA, is required for cystatin actions on B16 melanoma cells.

Introduction

Metastatic melanoma is an aggressive disease that is characterized by rapid systemic dissemination. The main cause of death in melanoma patients is widespread metastases. If caught early and treated before melanoma is able to metastasize the 5-year survival rate is greater than 95%, however, patients with metastatic melanoma have a 5-year survival of less than 10% (Tas 2012). Progression of melanoma involves a critical change from low metastatic, radial phase growth to higher metastatic, vertical phase growth. This is the basis for the prognostic value of the Breslow index for melanoma. As melanoma progresses, the cells invade through the basement membrane into the dermis and eventually into the peripheral lymphatic vessels and vasculature. A chief feature of invasive melanoma cells is the increased production of extracellular proteases for extracellular matrix (ECM) degradation. Several families of proteolytic enzymes are involved in tumor cell ECM degradation including MMPs, cathepsins, and plasminogen activator types in particular (Mason & Joyce 2011, 2012, Tan *et al.* 2013). In addition to their role in ECM degradation, the cathepsins also have roles in tumor cell proliferation and angiogenesis (Gocheva *et al.* 2006). Since several cathepsins are upregulated in most malignant tumor types, their inhibition may be of therapeutic benefit to control certain cancer metastases.

Cystatin C, a widely expressed cysteine protease inhibitor of about 14kD, has been shown to be a "metastasis suppressor" when overexpressed in cancer cells or in mice (Cox *et al.* 1999, Kopitz *et al.* 2005, Wegiel *et al.* 2009). Overexpression of cystatin C in murine melanoma cells results in decreased cell migration and metastasis (Ervin & Cox 2005). While the decreased metastasis upon cystatin C overexpression could be related to the decreased melanoma cell migration/invasion, it is more likely due to the increased apoptosis observed in melanoma metastases. This is because the growth of metastases at secondary sites is believed to be the metastasis rate limiting step (Chambers *et al.* 2001). The mechanisms for these effects are not well understood.

Considering that 1) overexpression of cystatin C results in decreased melanoma migration and metastasis; 2) exact sequences for cathepsin B inhibition by cystatin C have been evaluated; and 3) complete inhibition of all cysteine proteases would probably not inhibit melanoma migration and metastasis to cystatin overexpression levels observed, we explored the possibility of cystatin C inhibiting metastasis and migration through a different functional pathway, besides acting as a cysteine protease inhibitor. Since the cystatin sequence QVVAG is evolutionarily conserved in all cystatins and plays a minor role in cysteine protease inhibition, we compared this region to an N-terminal 10 amino acid cystatin deletion which is deficient for cysteine protease binding and inhibition (Abrahamson *et al.* 1991, Jerala *et al.* 1990). We analyzed expression of loss of function mutation of QLVA in murine cystatin C and, in a second mutation, the first 10 amino acid deletion of cystatin on migration, invasion, proliferation and cellular morphology in B16F10 (high metastatic) melanoma cells.

Materials and Methods

Cell line and Growth

B16F10 melanoma cells were obtained from ATCC. Cells were grown in RPMI1640 medium (Cambrex) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 1% stock antibiotic solution (100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin) (Sigma Chemical).

Cystatin C overexpression clone

Total RNA was isolated from B16F10 melanoma cells to be used for the production of murine cystatin C cDNA expression. Reverse transcription of the RNA was performed with MMTV reverse transcriptase and a 3' cystatin C primer. PCR amplification with Taq polymerase of the reverse transcriptase reaction was with 5' and 3' cystatin C primers. The PCR product was gel purified and ligated into an EcoRV-cut pcDNA3 plasmid for expression. Commercial sequence analysis showed a murine cystatin C sequence identical to that reported in the literature (Huh *et al.* 1995). The purified cystatin-pcDNA3 plasmid DNA was transfected into B16F10 melanoma cells with lipofectamine according to the manufacturer's instructions. Isolated clones were selected with cloning rings, following transfected cell colony growth in 1mg/ml G418. Cystatin overexpression clones were selected by western blot analysis after a 48-hour growth in serum free media. Samples were run on 12% SDS-PAGE gels and electro-blotted to PVDF membranes. Anti-cystatin C goat polyclonal antibody (Pierce) was used at 1:1000 dilution. Secondary antibody, rabbit anti-goat IR Dye,800 CW (Li-COR) was used at 1:10,000 and detection was performed by the Odyssey infrared imager (Li-COR).

Cystatin C Variant Clones

The clones used in this study consisted of a vector control (PD-1), a cystatin C overexpressing clone (CC12), two N-terminal 10 amino acid deleted cystatin C clones (1-1, 1-7) and two QLAV to GGGG (position 55-58) clones (4-3, 4-6). Double-stranded, sequence-verified genomic blocks of each cystatin variant clone were ordered (gBlocks® Integrated DNA Technologies). The Expresso™ CMV Cloning and Expression System from Lucigen® was used for rapid cloning of the cystatin construct clones into the pME-HA plasmid. A C-terminal human influenza hemagglutinin tag (HA) was used for detection of expression of the cystatin construct clones by B16F10 melanoma cells via western blotting. Each cystatin C variant clone was transfected into B16F10 cells with Lipofectamine (Invitrogen) according to the manufacturor's instructions. Lipofectamine solution was removed and complete growth media was added to the cells for overnight incubation. The cells were then trypsinized and seeded on 60mm dishes containing 1 mg/ml Geneticin (Sigma) for 10-14 days. Geneticin resistant colonies were isolated with glass cloning rings and 3-5 minute trypsinization with 50 µl trypsin solution, before moving cell colonies to 24-well plates containing 1 ml growth medium in each well.

Verification of Cystatin C Variant Clones

Western blots were perfomed to identify expression of cystatin C variant clones based on the intensity of the HA tag band. All clones were grown to near confluence in 24 well plates in complete RPMI medium and then switched to serum free RPMI for 3 days. Media (25µl) from each clone was then boiled for 5 minutes followed by centrifugation at 5,000 rpm. Samples were then ran on 12% SDS PAGE gels and electro-blotted to PVDF membranes for antibody detection. Rabbit anti-HA (Upstate Biotechnology) was used at 1:1000 dilution. Secondary antibody, goat anti-rabbit IR Dye,800CW (Li-COR) was used at 1:10,000 and detection was by Odyssey infrared imager (Li-COR).

MTT Assay

For each clone, $5x10^3$ cells in 100 µl of RPMI complete medium were plated per 96 well for a total of 3 wells per clone. The experiment consisted of wells seeded with cells for 0, 24, 48 and 72 hour time points. At each time point, media was taken off and replaced with 10% MTT stock solution (5mg/ml) with 90%

complete RPMI media, followed by incubation at 5% CO_2 and 37^0 C for 4 hours. After incubation, the solution was taken off the cells and 100 µl of isopropanol was added. The plate was then placed on a plate rocker at 30x speed for 15 minutes, foil covered. Absorption readings were measured, immediately after rocking, at 570 nm. Net proliferation was graphed from the relative absorption change divided by the absorption at time zero for each clone and variant clone. Each assay was performed in triplicate, and data is presented as mean ± standard error.

Wound Closure Migration Assay (Scratch Assay)

A reference line was drawn on the bottom of a 24-well plate through each well with a fine point permanent marker before plating cells. Cells at a density of $4x10^5$ cells per 2 ml were first plated in a 24-well plate for 12 hours in serum-containing medium and then an additional 12 hours with serum-free medium. Cells were then scratched in a straight line, perpendicular to the reference line, with a 200 µl yellow pipette tip in three places in order to create cell-free stripes (scratches). Media from each well was replaced after all three scratches were made. Digital images were taken immediately after performing the scratches and after 24 hours using an Olympus inverted microscope (4x objective). Digital images of cells on the glass coverslips were captured with a Motic $_{TM}$ digital camera and Motic images plus ML application software (version 2.0). When using the 4x objective, the field-of-view was 1875 µm x 1500 µm at the detection surface of the camera. Cell migration was measured by change in scratch-wound closure by the following formula**:** ((Time zero area - Time 24hr area)/ (2*307))/24).

Modified Boyden Chamber

Invasion was assessed with a modified Boyden chamber (Neuro Probe Inc., Pleasanton, CA) and 8 µm pore size polyvinylpyrrolidone (PVP)-free polycarbonate filters (Neuro Probe Inc., Pleasanton, CA). The filters were coated by pipetting 50 µl Matrigel solution (BD Matrigel^{TM}) evenly on top. The lower compartment of the chamber was filled with complete media plus chemoattractant, 25ng bFGF. The upper and lower compartments were separated by a Matrigel-coated filter. Melanoma cells (70-90% confluent) were harvested in 0.25% tryspin-EDTA solution (3 minutes), collected by centrifugation, resuspended in complete media and placed in the upper compartment ($3x10^4$ cells/100 µl per well). The invasion assay was performed in triplicate for each clone. After 24 hours of incubation at 37ºC, cells on filters were fixed in methanol for 10 minutes, permeabilized for 2 minutes with 0.1% Triton in PBS, and stained with 2% crystal violet for 15 minutes. Cells on the top portion of the membrane were carefully removed with a cotton tip swab before mounting on a glass slide with 70% glycerol solution. Invading cells were counted using microscopy and entire filter was analyzed for invading cells. To verify accurate counting the Image J cell counter plugin (NIH) was also used. Microscopy was performed with an Olympus inverted microscope (4x objective).

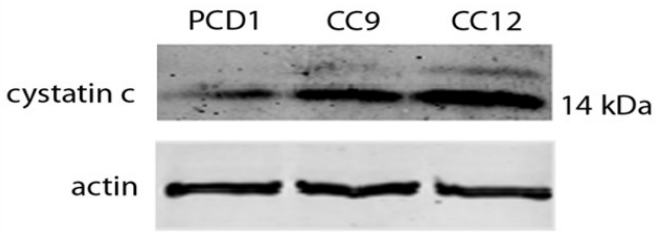


Figure 1. Western blot analysis for cystatin C overexpression clones. Two independent B16F10 melanoma cell clones were analyzed for cystatin C production from serum free media by western blot as described in Materials and Methods section. PCD1 is the pcDNA3 plasmid clone 1; CC9 and CC12 are the cystatin C overexpression clones.

Cell Shape

Aspect ratio (elongation), feret diameter maximum and minimum, roundness, area, perimeter and circumference for B16F10, vector clone PCD-1, cystatin clone CC12, clones 1-7 (N-terminal deletion) and 4-3 (QLVA>GGGG) were analyzed using image J (NIH). Each of the clones was sparsely plated ($5x10^4$ cells/35mm dish) onto 3 glass coverslips per dish per clone. After cells had flattened (6 hours), digital images were taken. Microscopy was performed with a Zeiss Axiovert 200M microscope using the 20x objective. Digital images of cells on the glass coverslips were captured with a Scion CFW-1310C digital camera and Scion VisiCapture application software (version 4.7). When using the 20x objective, the field-of-view was 145 x 130 µm at the detection surface of the camera. Images included 5 random fields for each coverslip and at least 100 cells were measured for each clone. Each assay was performed in triplicate, and data

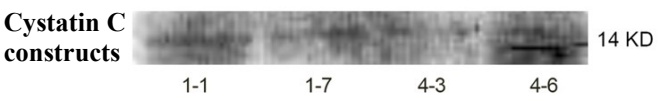


Figure 2. Western blot analysis of cystatin C variant clones. N-terminal deletion (1-1 & 1-7) and QLVA > GGGG (4-3 & 4-6) clones were analyzed for HA-antigen expression from serum free media by western blot as described in Materials and Methods section.

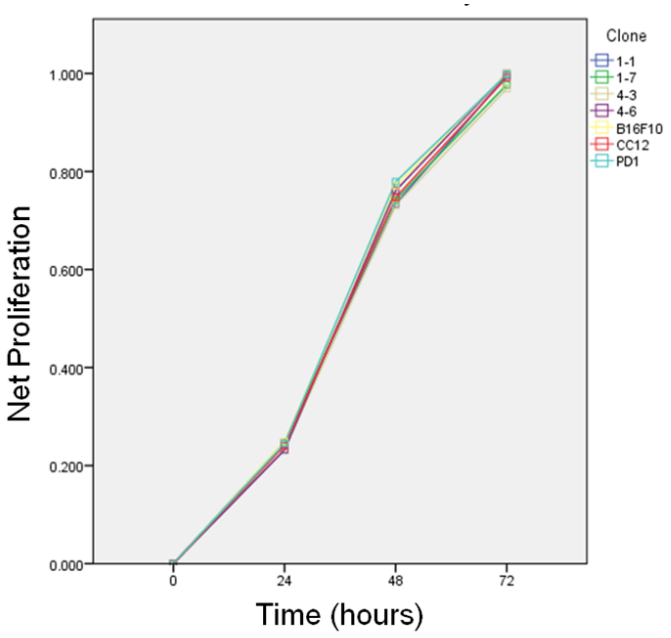


Figure 3. Net cell proliferation for B16F10 and variant clones as determined by MTT assay.

is presented as mean ± standard error.

Data Analysis

Data were analyzed for statistical significance using an Analysis of Variance and a Tukey post-hoc multiple comparisons test. A general linear regression model was used to test for differences in cell morphology due to experimental design. Differences corresponding to $p \leq .05$ were considered statistically significant.

Results

Cystatin C overexpression in B16F10 melanoma

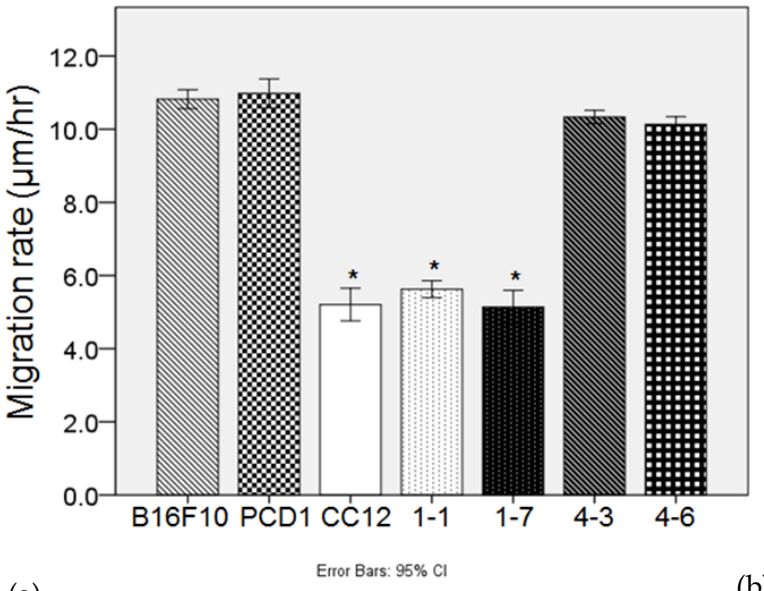
Murine cystatin C was overexpressed in B16F10 melanoma cells to serve as a control for cystatin variant clones. An increased level of secreted, 14kD cystatin C was detected for two overexpression clones (CC9 and CC12) by western blot analysis after a 48-hour culture in serum-free media (Figure 1).

Western blot of cystatin C variant clones

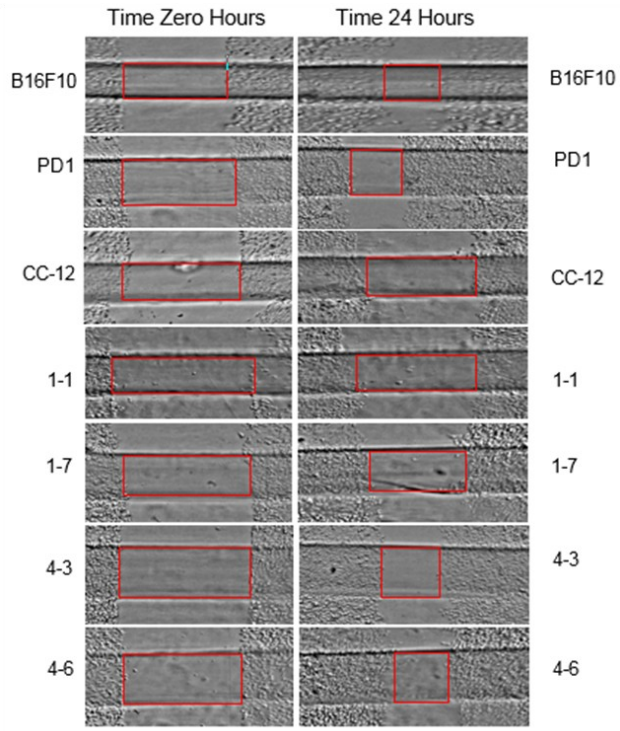
We aimed to establish what effects overexpression of cystatin C variants might have on the B16F10 *in vitro* behavior. To distinguish cystatin variant clone expression from endogenous cystatin C expression, we fused the variant cystatin constructs to HA tags. Western blot analysis after 48 hour, serum free culture of clones set 1 (N-terminal deletion) and 4 (QLVA to GGGG) allowed for detection of expression clones based on HA tag expression (Figure 2). The size of the expressed cystatin variants corresponded to the size of cystatin C (14kD).

Cell proliferation assay

To determine if cell proliferation rates were being altered by overexpression of cystatin C constructs, we



(a)



(b)

Figure 4. (A) Melanoma cell migration determined by scratch assay. Scratches which denuded cells were made in cell plate wells. Cells were allowed to migrate for 24 hours before images were taken under bright field microscopy. Vector control = PCD1, WT cystatin = CC12 cystatin overexpressor. (B) Microscopy of scratch assays for cell migration for all clones tested at zero and twenty four hours.

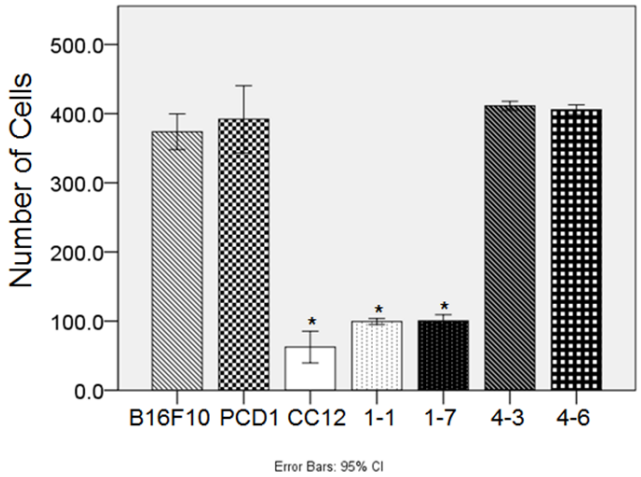


Figure 5. Melanoma cell invasion determined by Boyden-type filter assay. Filters with 8µm pores were used in the assay. Stained cells were counted under bright field microscopy. Vector control = PCD1, WT cystatin = CC12 cystatin overexpressor.

measured net proliferation of all clones using an MTT assay. There were no differences over time between each of the clones when measuring net proliferation rates (Figure 3). Therefore, clones of cystatin C expression constructs are not altered in net cell proliferation rates which could otherwise interfere with other results determined in this study.

N-truncated cystatin C clone mimics CC12 in cell migration rates

To evaluate if cysteine protease inhibition is crucial for decreased migration *in vitro* of B16F10 cells, scratch assays were performed with each clone. Figure 4 shows the results of scratch migration assay for each clone. Mean migration rates in µm/hr were calculated for each clone. Migration rates were similar between the cystatin overexpression clone (CC12) and N-terminal deletion clones 1-1 and 1-7. Migration rates were significantly different between the N-truncated clones 1-1 and 1-7 compared to the parental B16F10, vector control clone PD-1 and GGGG substitution clones 4-3 and 4-6. The QLVA to GGGG cystatin C construct clones mimicked B16F10 cell migration rates for 24 hours.

N-truncated cystatin clones have equivalent anti-invasion effects as CC12

In order to evaluate changes in invasion for the different cystatin clones, a modified Boyden chamber assay was implemented. There was a significant difference between CC12, 1-1 and 1-7 invasions when compared to the other clones. Clones 1-1 (99 cells), 1-7 (100 cells) and CC12 (62 cells) were significantly lower than B16F10 (374 cells), PD-1 (392 cells), 4-3 (411 cells) and 4-6 (406 cells). There was approximately a 4 -fold difference in invasion between clones 1 set and clones 4 set (Figure 5). The reduction in cell invasion through the membrane filter for N-truncated cystatin clones demonstrates how, even with cysteine protease inhibition activity missing for cystatin, there is still inhibition of invasion relative to control clones.

Cellular morphology of N-truncated cystatin and GGGG substitution clones

To determine cellular morphological changes between the different clones, cells of each clone were imaged with light microscopy with a 20x objective and analyzed with Image J. Feret diameter size, area, perimeter and circumference were not different between the clones ($p > 0.05$). Aspect ratio and roundness were, however, significantly different between the clones (Figure 6a and b). B16F10 cells were less elongated and more round than the cystatin overexpression clone (CC12). The mean aspect ratio and mean roundness for B16F10 compared to CC12 was 2.82 vs. 4.86 and 0.43 vs. 0.28, respectively. A larger aspect ratio translates to a more elongated cell. With a roundness of 0.43, B16F10 is much more round compared to CC12 based on a scale of 0 to 1, with 1 being completely round. N-terminal deletion (clone 1-7) and GGGG clone (clone 4-6) were also analyzed for cell morphology. Clone 1-7 had comparable aspect ratio and roundness as CC12, and both were statistically different from clone 4-6 and B16F10 values. Clone 4-6 showed aspect ratio and roundness values similar to B16F10. Mean aspect ratio and mean roundness for clones 1-7 and 4-6 were 4.57, 2.63 and 0.29, 0.44 respectively.

Discussion

The goal of this study was to study the effects of two different cystatin C construct clones on B16F10 *in vitro* cell behavior by evaluating cell proliferation, migration, invasion and cell morphology. Previous work has shown cystatin C overexpression inhibits the metastasis of B16 melanoma (Cox *et al.* 1999). Key changes induced by cystatin overexpression in the metastasis of melanoma cells are both inhibition of cell migration/invasion and higher apoptosis induction *in vivo* (Ervin & Cox 2005). We initiated this study with a question as to whether melanoma cell migration/invasion was inhibited by the cysteine protease inhibitor action of cystatin. Two key different cystatin construct clones were compared in their *in vitro* behavior to parental clones and cystatin overexpression clone – N-terminal cystatin deletion (not cathepsin inhibitory) and QLVA to GGGG construct. Net cell proliferation was not significantly different between control clones

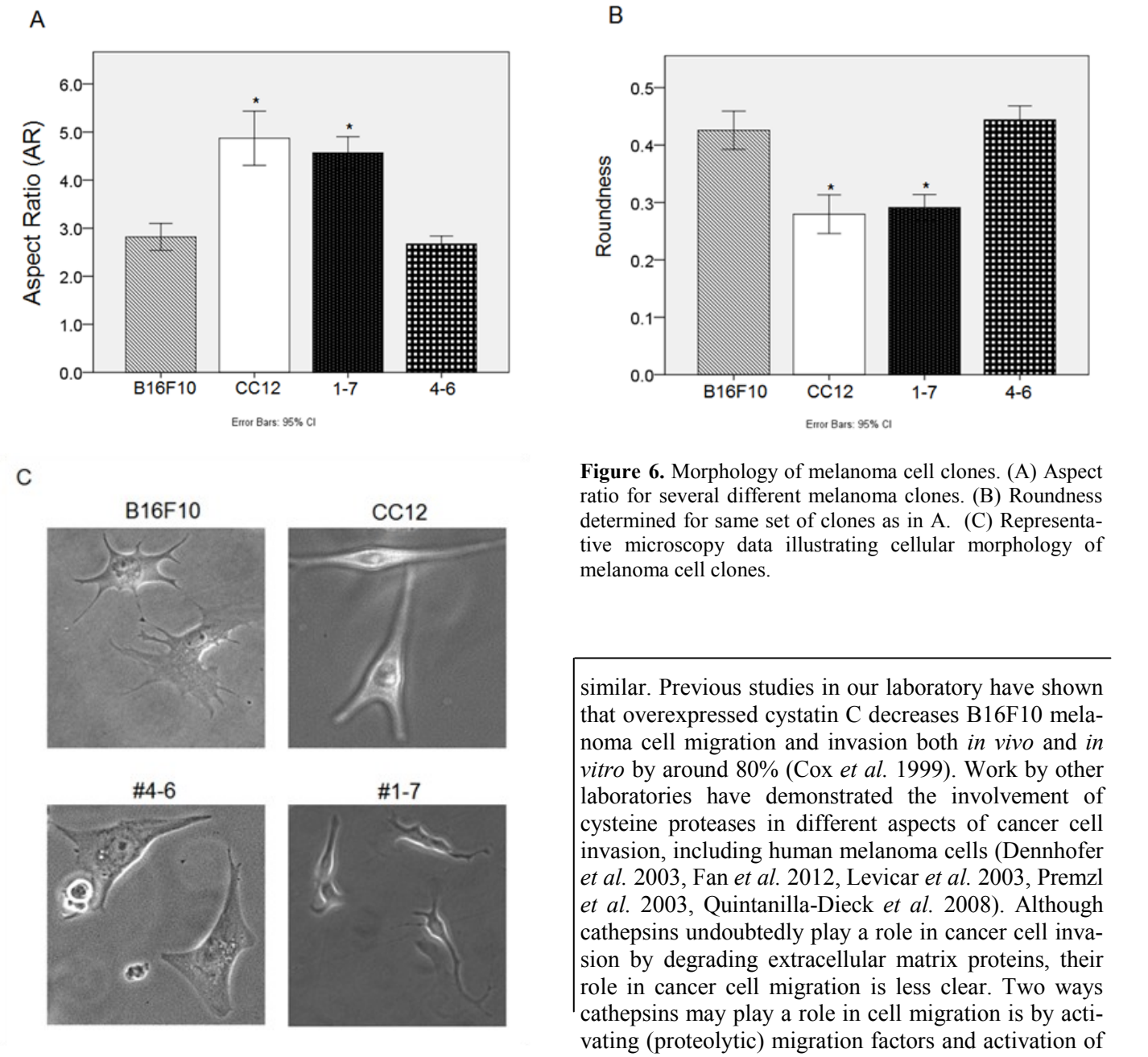


Figure 6. Morphology of melanoma cell clones. (A) Aspect ratio for several different melanoma clones. (B) Roundness determined for same set of clones as in A. (C) Representative microscopy data illustrating cellular morphology of melanoma cell clones.

and the cystatin overexpression or cystatin C variant clones *in vitro*. This result differs from the *in vivo* results of increased apoptosis with cystatin C overexpression and might be due to the growth factor-rich environment which exists *in vitro*. Alternatively, the concentration of secreted cystatin in cell culture may be insufficient to increase cell apoptosis as seen *in vivo*. We found that the evolutionarily conserved cystatin QLVA sequence is necessary for obtaining cystatin anti-migratory behavior when over-expressed in B16F10 cell line *in vitro* based on loss of function for QLVA variant clones.

Since the N-truncated cystatin clones had migration and invasion rates comparable to wild type cystatin clone, the mechanism of inhibition should be similar. Previous studies in our laboratory have shown that overexpressed cystatin C decreases B16F10 melanoma cell migration and invasion both *in vivo* and *in vitro* by around 80% (Cox *et al.* 1999). Work by other laboratories have demonstrated the involvement of cysteine proteases in different aspects of cancer cell invasion, including human melanoma cells (Dennhofer *et al.* 2003, Fan *et al.* 2012, Levicar *et al.* 2003, Premzl *et al.* 2003, Quintanilla-Dieck *et al.* 2008). Although cathepsins undoubtedly play a role in cancer cell invasion by degrading extracellular matrix proteins, their role in cancer cell migration is less clear. Two ways cathepsins may play a role in cell migration is by activating (proteolytic) migration factors and activation of migration related genes (non-proteolytic). Due to the first 10 amino acids of cystatin C being necessary for cysteine protease inhibition and binding, our findings suggest cysteine protease inhibition is not the main mechanism by which cystatin C is inhibiting melanoma migration, invasion and metastasis.

Migration of melanoma cells resembles a snail -like crawling involving focal adhesions in the case of epithelial to mesenchymal transition (EMT) or a weakly attached and gliding movement seen in amoeboid-style mode (Wolf *et al.* 2003). In EMT migration, cells exhibit an elongated morphology versus a rounder shape in the amoeboid-type. B16F10 cells were morphologically very different compared to the cystatin C overexpression and N-terminal deleted

clones. B16F10 cells had more filopodia-like structures and were less elongated. Filopodia have been shown to play a role in cell migration as protrusions containing actin cytoskeletal elements (Lidke *et al.* 2005). The cystatin overexpression and N-truncated cystatin clones appeared to be very elongated, suggesting reduced cell ability to undergo rear detachment from the surface and allow migration. Calpains are cysteine proteases involved in cellular migration through mediating rear-end cellular detachment (Glading *et al.* 2002). Cystatins are not known to inhibit calpains, but the effect of cystatin inhibition of cell migration mimics the cell elongation effect that might be seen, for example, with calpain inhibition. More work will be required to determine why cystatin over-expression results in cancer cell elongation as it relates to decreased cell migration.

Progression of melanoma from the primary tumor to a secondary site involves degradation and invasion through the basement membrane and angiogenesis. Our results from the cell invasion assay demonstrate that without the QLVA region, cystatin C cannot inhibit B16F10 invasion, whereas deletion of the first 10 amino acids has little impact on decreasing cystatin C inhibition of B16F10 invasion. We propose that the QLVA region is interfering with some cell signaling pathway required for melanoma cell migration/ invasion. In a prostate cancer cell line (PC3), cystatin C was shown to act as a factor that inhibited MAPK/ Erk2 pathway (Wegiel *et al.* 2009). MAPK signaling plays an important role in degrading the basement membrane, enhancing migration and maintaining tumor cell growth (Sumimoto *et al.* 2004). We have not yet determined whether MAPK signaling may be linked to cystatin inhibition of invasion in B16 melanoma cells.

The major conclusion of our study is that QLVA is the sequence of cystatin C necessary for its inhibition of migration and invasion for B16F10 cells. The cysteine proteinase inhibitor activity of cystatin C is not involved in inhibition of B16F10 invasion, but some other signaling pathway is probably being regulated which contributes to the majority of the inhibition in B16F10 invasion and migration by cystatin C overexpression.

Competing interests

The authors declare no conflict of interest concerning this work.

Authors' contributions

SM did all the experiments involving cells and data analysis and assisted with manuscript preparation. JC produced the melanoma clones, assisted in some experiments, and wrote major portions of the manuscript.

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