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Full Paper

Isolation of circulating tumor cells to diagnose melanoma and evaluate the efficacy of surgical resection using melanoma-specific microsystem

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Abstract

Melanoma is one of the most aggressive skin cancers due to its potential to metastasize widely in the body. The risk of metastasis is increased with later detection and increased thickness of the primary lesion, thus early identification and surgical removal is critical for higher survival rates for patients. However, even with appropriate treatment, some patients will develop recurrence which may be difficult to identify until advanced or causing symptoms. Recent advances in liquid biopsy have proposed less-invasive alternatives for cancer diagnosis and monitoring using minimal/zero invasion at sample collection, and circulating tumor cells (CTCs) have been considered a promising blood-based surrogate marker of primary tumors. However, previous CTC technologies relying on epithelial-cell adhesion molecules have limited to epithelial cells, thus hampering use of CTCs for non-epithelial cancers such as melanoma. Here, we used the Melanoma-specific OncoBean platform (MelanoBean) conjugated with melanoma specific antibodies (MCAM and MCSP). The device was used in comprehensive studies for diagnosing melanoma and evaluating surgery efficacy based on change in the number and characteristics of CTCs and CTC-clusters pre- and post-surgical treatment. Our study demonstrated that melanoma patients ($n=45$) at all stages (I-IV) have a noticeable number of MCTCs as well as MCTC-clusters compared to healthy donors ($n=9$) ($P=0.0011$), and surgical treatment leads to a significant decrease in the number of CTCs ($P<0.0001$). The CTCs recovered from the device underwent molecular profiling for melanoma-associated genes expression using multiplexed qRT-PCR, demonstrating the ability to monitor molecular signature through treatment. The presented MelanoBean and the comprehensive approach will empower prognostic value of CTCs in melanoma in much larger cohort studies.

1. Introduction

Melanoma is potentially lethal form of skin cancer whose incidence rate continues to increase, with over 300,000 new cases reported globally in 2019^[1]. Once a patient develops metastatic melanoma, historically few options existed for treatment^[2, 3]. With the advent of newer targeted therapy options and several immunotherapy options, patients with advanced or metastatic melanoma have more options and increased responses to therapy, and overall survival has increased^[4].

Some melanomas can be removed surgically, and after surgery, patients have seen significant increases in survival rates^[5-7]. Following optimal surgical management of the primary lesion patients at higher risk of recurrence are generally monitored closely with a combination of physical exam, ultrasound and possibly other radiographic techniques such as CT scans, PET scans or MRI. However, additional options for monitoring for recurrence are of great interest, particularly methods that avoid the risk of additional radiation exposure from repeat imaging. One method that has gained significant traction in this regard is liquid biopsy^[8].

Liquid biopsy is a less-invasive sampling of biomarkers from bodily fluids such as blood and plasma. This technique has garnered significant attention as a cheaper, faster, and less invasive alternative to traditional tissue biopsy^[9, 10]. While applicable to a variety of circulating markers, circulating tumor cells (CTCs) have emerged as a metastatic precursor and also known to have molecular information about the tumor, thereby enabling metabolomics, proteomic, genomic, and transcriptomic studies of cancers^[11]. Recent studies have shown that the concentration of CTCs in patient's blood is correlated with survival and prognosis in several forms of cancer^[12, 13]. However, the low concentration of CTCs (1-10 cells per milliliter of whole blood) typically found in cancer patients has made sensitive detection techniques critical for successful liquid biopsy^[12].

In spite of major efforts and obvious potential of using CTCs, all existing CTC assays including the only FDA-cleared CTC platform, CellSearch, are not recommended for use in melanoma diagnosis because of low sensitivity and inconsistent results^[14]. Recently, several liquid biopsy strategies

have been developed to monitor CTCs in patients, such as filtration [15-17], gradient based centrifugation [18, 19], hydrogel-based isolation [20], in-vivo photoacoustic flow cytometry based platform [21], and microfluidic platforms [22-24]. Of these platforms, microfluidics offers the most potential for CTCs due to their extremely sensitive detection and isolation while maintaining minimal sample loss and high throughput processing conditions. Specifically, microfluidic devices incorporating immunoaffinity-based CTC isolation strategies [25, 26] have shown noteworthy reliability, repeatability and sensitivity with a merit of on-demand choice of antibodies to be used for CTC capture [27].

While numerous studies have been conducted investigating the role of CTCs and epithelial cancers, few have examined the prognostic potential of CTCs in melanoma. Using CTC filtration system, De Giorgi et al. found that CTCs can be found in the initial stages of melanoma progression and may be detected in 29% of patients with primary invasive melanoma and in 62.5% of metastatic melanoma patients [28]. Semi-automated immunoaffinity-based CellSearch with melanoma kit has been used for melanoma studies [29-32] and Lucci et al. found that melanoma CTCs could be identified in 37% of stage III melanoma patients, and that the presence of at least a single CTC in these patients was independently associated with melanoma relapse [29]. Hall et al. used the same system with modifications to determine that for patients with stage IV melanoma, the presence of 1 or more CTCs in an initial sampling was indicative of disease progression within 180 days [32]. While these studies demonstrate the clinical potential of CTCs in disease monitoring, the low sensitivity and detection rate of melanoma CTCs has limited further translational/downstream analysis. Thus, they emphasize the need for more sensitive detection methods for melanoma CTC and further in-depth study to make definitive determinations using more sensitive technology.

One area requiring further study is in determining the relevance of CTCs in monitoring treatment response in melanoma patients. Liquid biopsy of CTCs in various cancers lends rapid insight into treatment response that allows physicians to guide treatment decisions. Pierga et al. found that the

quantity of CTCs in metastatic breast cancer patients was reduced at cycle 2 of first-line chemotherapy with targeted therapy^[33]. This demonstrates the prognostic utility of CTCs in cancer^[34]. In limited studies, comparing the count of CTCs before and during melanoma treatment has shown promising results with respect to cancer prognosis and therapy response in melanoma^[35-37]. These previous studies demonstrate that measuring changes in CTC numbers during treatment can be useful for monitoring therapy response and predicting survival in melanoma patients.

For applications in treatment monitoring, we investigated melanoma CTC quantification and molecular profiling using a novel microfluidic device, MelanoBean (MB) chip. The MB chip is a newly optimized platform of the previously reported OncoBean microfluidic device for melanoma^[38]. Thanks to its ability to process both large and small sample volumes at high throughput conditions, we applied this technology to a clinical study using blood samples from melanoma patients. In differentiating our MB device from the OncoBean, as well as tailoring it for melanoma CTC isolation, we functionalized this high throughput isolation device with antibodies specific to a subset of melanoma markers (MCAM and MCSP), allowing for isolation based on melanoma surface proteins. The bean-shaped posts allow for increased isolation of CTC clusters for further in-depth investigation towards CTC cluster in cancer^[39].

Using whole blood from melanoma cancer patients, we isolated melanoma circulating tumor cells (MCTCs) and CTC clusters for liquid biopsy. Our goal was to isolate, quantify, and analyze the genetic markers of MCTCs before and after melanoma surgical treatment to evaluate the efficacy of surgical treatment (**Fig. 1**). Furthermore, we hypothesized that a difference in MCTC quantity or MCTC-RNA expression after surgery could indicate increased likelihood for recurrence.

2. Results and discussion

2.1 MCTC isolation using MelanoBean microfluidic device

MelanoBean chips were fabricated using a standard soft-lithography method following previously defined procedures^[40] and functionalized with antibodies against MCAM and MCSP (**Fig. 2A**). The bean-shaped microposts are radially placed in the MB chip for effective MCTC isolation at high throughput conditions. To perform both enumeration and transcriptomic analysis of MCTCs, each clinical sample was applied to two identical devices following two distinct processing protocols (**Fig. 2B**, left). To confirm the ability of the MelanoBean chip to isolate MCTCs, we first used the melanoma cell line, SK-MEL-103, as a model MCTC sample and applied to the MB chip. The cells were pre-stained with the fluorescence stain CellTracker green to allow for quantification using fluorescence microscopy. As shown in **Figure 2B** right, a spiked melanoma cell was captured on a bean-shaped micropost in the device. We demonstrated that the MB device successfully isolates an average of $71.2 \pm 10.9\%$ of spiked cells at the concentration of 200-1,000 cells per milliliter of blood.

With the knowledge that our device can successfully isolate melanoma cell lines such as SK-Mel 103^[40], we continued with our isolation of the circulating MCTCs from melanoma patients. Two 3 ml samples of blood drawn from melanoma patients both before and after surgical resection were applied to separate two MCAM/MCSP functionalized MelanoBean devices.

One pre-surgery and one-post-surgery device were fluorescently stained for enumeration while the second processed pre- and post-surgery MCTCs were lysed with RLT buffer for RNA analysis, see below. For enumeration, isolated MCTCs were then stained with a primary antibody cocktail of DAPI, CD-45 (*FITC*), and s100/MelanA (*CY3*) using fluorescent dyes for quantification by fluorescence microscopy. The presence of DAPI and s100/MelanA positive and CD-45 negative cells demonstrates that MelanoBean devices successfully isolates MCTCs on the surface of the

PDMS bean-shaped posts. Fluorescent images in **Figure 2C** confirm MCTCs from a patient sample are successfully isolated on the device surface.

2.2 Strategy for MCTC analysis and MCTC counts before and after surgery

Surgical treatment, including wide local excision, is the primary treatment option for most melanomas. Monitoring CTC changes to evaluate treatment efficacy offers a possible approach for rapid patient monitoring that is critical for patient care. In a proof-of-concept study, Juratli et al. discovered that a proper tumor resection of melanomas significantly decreased CTC counts using melanoma bearing mice ^[41]. Quaglino et al. showed that CTC positivity was associated with development of new metastatic sites in patients who had undergone the surgical treatment, implying potential clinical use of melanoma CTCs. ^[42] To demonstrate this monitoring using CTCs, we collected two different blood samples from each patient. Preoperative peripheral (Pre-Op) blood was collected 1-2 hours prior to surgery. Postoperative blood was drawn via a peripheral vein at the time of surgery and both pre- and post-op blood samples were collected in EDTA tubes and processed on the same day of surgery for the MCTC enumeration and RNA extraction. Whole blood samples from healthy donors were also collected for control experiments and processed using the same platform. As both pre/post blood volumes were most often limited to 3 mL of blood, MCTC numbers are reported as MCTCs normalized per 3 mL.

Enumeration of MCTCs isolated on-chip was performed by imaging and analyzing the entire device surface following fluorescent staining (**Figure 3A**). Selection criteria for positive identification of MCTCs include shape, size, and fluorescence (DAPI+/CY3+/FITC-). In total, 45 melanoma patients were examined before and after surgery, along with 9 healthy donors. We compared the MCTC concentration from both melanoma patients (n=45, pre-op) and healthy donors (n=9). We found that all 45 patients were MCTC positive (contained ≥ 1 MCTC) before surgery, while 6 out

of 9 healthy donors contained any MCTC marker positive cells ranging 1-5/3mL. Average MCTC counts in patient samples (pre-op) were significantly higher, 14.64 ± 11.35 MCTCs/3mL blood while healthy donors averaged just 1.44 ± 1.81 MCTCs/3mL blood (p -val=0.0011) (**Figure 3B**). Next, we compared the MCTC numbers isolated from patients at pre- and post-op (**Figure 3C**). Following surgery, the number of patients that were MCTC positive dropped from 100% to **84.4%**, and average CTC count dropped to 0-22 CTCs (average=5.27) from 1-51 CTCs (average=14.64) (p -val<0.0001, unpaired t-test) (**Figure 3D**). Results of pair-wise total CTC quantities found in each patient using this method are shown in **Figure 3E**. There was no correlation between the numbers of MCTCs at pre-op compared to post-op. As shown in **Figure 3F**, 38 of 45 patients displayed a decrease in MCTCs following surgery (p -val<0.0001, paired t-test). We next evaluated whether changes in the MCTC number after surgery is predictive of patient response to surgery (**Fig. S1**). The change slope was calculated for each patient. This slope was used as an indicator of MCTC changes during the surgery. In most cases, the number of MCTCs detected following surgical intervention decreased, implying that MCTCs can be used to evaluate the efficacy of the melanoma surgery.

Among 7 patients that did not show a reduction in MCTCs, 2 had no change, while 5 patients exhibited an increase in overall MCTCs. This significant overall decrease in the number of CTCs found in the blood of patients following surgical resection is in line with previously reported findings, such as those made by Jiao et al. who found that open resection of primary tumors in patients with colorectal cancer led to immediate reduction of CTC levels ^[43] while one of other studies using photoacoustic flow cytometry claimed that circulation time of CTCs after intervention is at least 100 minutes ^[44]. Real time CTC monitoring study showed that melanoma surgery reduces CTC counts, while pressure, biopsy, and laser-assisted cancer treatment increase CTC counts ^[41]. It is also noteworthy that no CTCs were detected in any mouse up to 3 hours after the tumor resection. Our findings are in accordance with these previous results from clinical human

samples and mouse models. At the same time, our results also led to needs for multiple samplings in the future study to track dynamic CTC changes at times.

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2.3. MCTC cluster counts before and after surgery

The presence and clinical implications of CTC clusters in melanoma and clinical implications has been studied recently from several in vivo studies [45, 46]. Thus far, CTC clusters have been detected in a few cancers, and studies indicate that they have a greater capability to be metastasized and are associated with poor disease prognosis [39, 47]. In this study, the presence of CTC clusters was also examined both before and after surgical intervention. Clusters were defined as a grouping of at least two CTCs within close proximity of each other that displayed multiple distinguishable stained nuclei under fluorescent microscopy. Results following cluster enumeration are displayed in **Figure 4** and representative immunofluorescence staining images of clusters are shown in **Fig. 4A**.

MCTC clusters were detected in over 15 % of the 45 patients irrespective of the surgery status, implying that CTC clusters may form in melanoma patients, similar to other cancers. We see that 48.9% (22 of 45) of the tested melanoma patients were MCTC cluster positive (contained ≥ 1 CTC cluster) before surgery (**Figure 4B**). Following surgery, we observe a sharp decline in the number of patients whose blood contains CTC clusters, with only 15.6% (7 of 45) of patients displaying at least a single CTC cluster (**Figure 4C**). None of the 9 healthy donor samples had clusters.

The average numbers in MCTCs found in clusters were 5.05 and 2.86 at pre-op and post-op, respectively. This reduction in CTC clusters following surgery has been previously reported in other cancers, as shown by Zhang et al., [48] although this study was regarding 3 month follow-ups. We also evaluated the number of CTC present as either in single CTCs (single form) or in CTC clusters (clustered form) (**Figure 4D-E**). In pre-op samples, 25.74 % of total CTCs were in single CTC form, with this number rising to 33.47% in post-op samples.

The clusters in pre-op samples varied in size from 2 MCTCs to 6 MCTCs, while those in either post-op group ranged from 2 to 3 MCTCs (**Fig 4F-G**). In the 45 pre-op patients, the average number of MCTCs in each cluster is 2.45, while single MCTCs averaged 12.18 (p -val <0.0001),

showing that still the major form of MCTCs is single MCTCs. This trend is also found in post-op samples where average MCTCs in cluster and single MCTCs form is 0.44 and 4.82 (p -val <0.0001), respectively. It is also noteworthy that of these clusters, more than 66% of MCTCs found in clusters are in clusters containing only 2 MCTCs in both pre- and post-cases even though various sizes of MCTC clusters have been reported previously. Further studies are needed for a clearer conclusion.

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2.4. RNA quantity comparison before and after surgery

We next examined RNA quantities from the isolated MCTCs within each patient. The second device that processed exactly the same sample was used for RNA quantity/gene expression analysis. After isolation of MCTCs, RLT buffer (Qiagen, USA) was applied to each device for lysis and extraction of RNA. These RNA samples were then analyzed for RNA concentration and therefore quantity using the Bioanalyzer (Agilent, USA). Results for RNA quantity in captured samples are shown in **Fig. 5A-B** and are normalized to show RNA per mL of blood for all cases. Overall, pre-op samples showed higher RNA quantities compared to post-op samples. This higher RNA concentration from pre-op might be correlated with higher MCTC numbers in our MCTC enumeration studies. Violin plot of mRNA quantities for pre-, post-surgery and healthy donors is described in **Figure 5C**. There were no significant differences between average RNA quantities in three different groups. However, pre-op samples showed high variance while the other two groups showed negligible variances, which might imply that significantly higher MCTC numbers in pre-op compared to other two yields this variance.

As shown in **Figure 5D**, 24/45 patients displayed a decrease of RNA quantity in MCTCs following surgery. One patient did not show a reduction in RNA quantity, while 20 patients exhibited an increase in overall RNA quantity. Compared to MCTC enumeration, RNA evaluation might be less sensitive to MCTC changes due to technical difficulties. As we extracted RNA right after MCTC isolation and some non-specific cells, such as leukocytes, can be lysed together, this might have led to total mRNA quantity increase. The extracted RNAs at both pre- and post-op were further qualitatively studied in terms of 96-melanoma associated gene expressions.

2.5. 96 gene expression profiling of captured MCTCs

Following isolation of MCTCs by MelanoBean device, both pre- and post- surgery patient samples were lysed for RNA extraction. The extracted RNA samples were further purified and subjected to multiplexed qRT-PCR. Gene expression profiles for each patient were then analyzed using SINGuLAR platform for 96 different genes. Pre- and post- operative gene expression results for each patient are displayed in **Figure 6A-b**. As we see from these results, several genes are highly expressed amongst most samples tested (**Fig.6A**). These genes include growth inhibitory gene (TGFB1), metastasis associated gene (CD29), inflammation associated genes (S100A8, S100A9), B-cell translocation gene (BTG1), immune escape gene (B2M), housekeeping genes (GAPDH, ACTB), EMT gene (ADAM10), and Parkinson's disease associated gene (SNCA). In addition to these highly expressed genes, we also see several genes that display little to no expression amongst all patients tested, both before and after surgery. These include KRT14, ROBO1, EGFR, CD133, and WNT5A.

While most samples tested displayed a diverse range of expression amongst the profiled genes, some samples showed significant uniformity in their overall expression. In particular, several samples showed little to no expression amongst all genes tested. For example, Patients 2 and 14 displayed little gene expression of note in their post-op samples, while patients 23 and 24 showed almost nothing in their pre-op sample profiles. Patients 24 and 27 displayed almost no gene expression in both their pre and post-surgery samples (**Fig. 6B**).

The generated 96 gene profiles from each patient's pre- and post- operation samples were then compared, as shown in **Figure 6C**. As shown by this heat-map, several genes had a notable change in expression following surgery amongst the cohort. First off, while CD44 appears to show significant expression amongst most tested samples, a decrease in its expression can be found in 16 of the 27 patients following surgery. CD44 gene and its isoform has been known to have correlations with tumor growth and metastasis, thus has been shown to lead to low survival rate^[49].

Similarly, vimentin was highly displayed in most samples, however, it showed some amount of decrease in 16 of the patients after the surgery, with 6 patients showing an increase in expression. This gene has been considered as a prominent biomarker in previous melanoma studies and its overexpression in cancer cells has shown to be correlated to increased tumor growth ^[50].

TGFB1 showed the most significant amount of decrease in expression following surgery, with a total of 17 patients showing less expression in their post-op profiles, while 5 patients showed no change. A similar alteration was found in the CXCL8 gene, where again, 17 out of 27 patients showed a decrease in expression.

Not all genes with notable expression changes were decreases following surgery. LIF, which in general showed very low expression amongst samples, demonstrated a slight increase in post-op expression. Only 2 of the 27 tested patients displayed any amount of LIF, however, following the operation, 7 patients expressed a small amount of the gene. Similarly, 9 of the patients' pre-op samples tested positive for any amount of the Pirin (PIR) gene, while 12 of the post-op profiles had some notable expression.

Some patients even showed overall opposite trends amongst almost all the genes tested in their profiles to others. Even amongst the genes that trended towards decreasing expression with surgery, patient 9 showed an increase in almost all 96 genes tested in their panel. In contrast to patient 9, patient 25 showed a decrease in all genes that showed any expression in their panel. These results give significant insight into the changing genetic expression induced by the surgical intervention on the isolated MCTCs.

Conclusion

In this study, we have evaluated circulating tumor cells as a prognostic tool of melanoma surgical treatment using a microfluidic device. The device was conjugated with two clinically relevant

melanoma antibodies, MCAM and MCSP antibodies, facilitating the sensitive isolation of MCTCs and MCTC clusters and demonstrating the high reliability of the current platform to be used in clinic. The specificity offered by the present immunoaffinity-based device showed that melanoma patients have more than 4.87 MCTCs per 1mL of whole blood, while healthy donors have less than 0.47 MCTCs/mL. The versatility of the present device in isolating both single MCTC and clustered MCTCs allowed us to demonstrate that surgical treatment significantly decreased the number of MCTCs as well as MCTC clusters for most melanoma patients. Furthermore, pre- and post-op blood samples were processed to evaluate melanoma associated gene expression changes in MCTCs. Despite the significant decrease in MCTC counts, the gene expression changes were not that noticeable. Perhaps, a larger cohort and a longer follow-up studies are needed for finding mRNA-based prognostic signatures in MCTCs.

The long-term goal of this project is to couple prediction of recurrence with the ability to provide valuable information about viable treatment options, perhaps through implementing systemic therapy that would remove metastatic precursor cells. Treatment plans could be personalized based on information gained from the liquid biopsy techniques. As such, the ability to isolate MCTC and MCTC clusters with high sensitivity while minimizing sample loss from melanoma patient blood samples empowers MCTCs to have a prognostic value as well as previously shown diagnostic values in many cancers, including melanoma.

3. Materials and Methods

Melanoma cell culture and model sample preparation: SK-MEL-103 cell line was used for preparation of our model sample. SK-MEL-103 cells were cultured in conditioned media, followed by spiking of 5,000 cells 1ml of whole blood sample. Cells were pre-stained by CellTracker dye (green) and isolated cells were evaluated using fluorescence microscopy.

Clinical sample collection and preparation: All experiments and sample collection was approved by Ethics committee (Institutional Review Board and Scientific Review Committee) of the University of Michigan with informed consents obtained from all participants of this clinical study. Melanoma blood samples were obtained after approval by the institutional review board at the University of Michigan (HUM00105509). All experiments were performed in accordance with the approved guidelines and regulations by the ethics committee at the University of Michigan. All patients were primary melanoma patients undergone surgical tumor removal and each whole blood sample was collected from the University of Michigan Rogel cancer center, with 6ml of whole blood collected before and after surgery used for CTC isolation. Pre-op samples were collected right before the surgery and post-op samples were collected within ½ hour of tumor removal. All samples were collected into K3 EDTA blood collection tubes (BD, USA) and CTC isolation was performed within 2 hours of blood collection.

MelanoBean chip fabrication and surface modification for melanoma: Previously defined procedures for the fabrication of MB chips were followed with modifications to allow for melanoma specific CTC capture^[34]. Briefly, O₂ plasma treatment (Covance, Femto Science, South Korea) was used to bond PDMS chambers to glass slides. To strengthen the bond, each device was placed on a hot plate at 80°C for 10 minutes. A 500µl silane in 5ml ethanol solution was then

applied to the inlet of each device and allowed to incubate for an hour. Unbound silane was removed by ethanol wash followed by application of GMBS cross linker solution (14 μ l GMBS in 5ml ethanol) which was allowed to incubate for 30 minutes before another ethanol wash. The injection of a 500 μ l NeutrAvidin in 5ml PBS solution was the final step before each device were parafilm sealed into humidified Petri dish containers and stored at 4°C awaiting future use. Upon sample acquisition, the stored MB devices were conjugated with melanoma CTC associated antibodies, MCAM (Mouse host, IgG1 κ , anti-human, Miltenyi Biotec, Germany) and MCSP (Mouse host, IgG1 κ , anti-human, Novus, USA). A 250 μ l antibody solution containing 2.5 μ l of anti-MCAM (2.5 μ g, 1mg/ml) and 2.76 μ l anti-MCSP (2.5 μ g, 0.91mg/ml) with the remainder 1% BSA was injected into each device and allowed to incubate for 1 hour. A PBS wash was then used to remove unbound antibodies, followed by blocking with a 3% BSA solution. Once blocking solution was washed out with a final PBS was, each device was properly functionalized to accept samples for CTC isolation.

Melanoma CTC and CTC cluster isolation: For CTC isolation, a PHD 2000 auto-pump (Harvard Apparatus, USA) was used to inject 3ml of whole blood into each antibody-conjugated device, MelanoBean chip, at a flow rate of 5ml/hr. Blood remaining in the devices was then immediately washed out using PBS at a flow rate of 10ml/h. Each device was then prepared for either DNA/RNA analysis or immunostaining for imaging.

Immunostaining of melanoma CTCs and CTC clusters: After blood samples were applied and washed out, devices designated for immunostaining were fixed with 1ml 4% PFA solution (250 μ l 16% PFA in 750 μ l PBS.) PFA fixation solution was allowed to incubate for 40 minutes before being washed out with PBS. Each device was then permeabilized with 1ml of 0.2% Triton solution

and incubated 30 minutes. Triton was then removed by PBS wash before the application of 1ml 3% BSA-2% normal goat serum solution (500 μ l 6%BSA, 200 μ l normal goat serum, 300 μ l PBS) which was incubated for 30 minutes. Primary staining antibody solution composed of 10 μ l anti-Melan-A/MART1 (R&D Systems, USA) 25 μ l S100 (mouse IgG2a, ThermoFischer, USA) and 25 μ l CD45 (rat IgG2b, Santa Cruz Biotech, USA) in 1ml of 1%BSA was pumped into each device and incubated for 1 hour. Excess primary antibody solution was removed by PBS wash. A secondary staining antibody solution was then applied containing 5 μ l AlexaFluor 546 (goat anti-mouse IgG2a, Life Technologies, USA) and 5 μ l AlexaFluor 488 (goat anti-rat IgG, Life Technologies, USA) in 1ml of 1%BSA and allowed to incubate for 1 hour in the dark. Excess secondary antibodies were removed by PBS wash followed by the application of 1ml DAPI staining solution (1 μ l DAPI in 1ml 1%BSA.) The DAPI solution was incubated for 15 minutes followed by a final PBS wash. Devices are imaged using *Ti2* microscope (Nikon, Japan) at 10x magnification for cell analysis. Images taken in FITC, DAPI and CY3 fluorescence.

Total nucleic acid extraction: Nucleic acid extraction is performed using RLT buffer (RLT Plus RNeasy Plus lyses, Qiagen, Germany). Device inlet and outlet tubing is removed and replaced with fresh 3” tubing. 50 μ l of RLT buffer is injected into inlet with the flow through collected in a sterile 1.5ml vial. Each device is then briefly vortexed. The RLT buffer injection and vortex is repeated twice more followed by the injection of air into the inlet to remove all liquid from the device. Collection vials are then stored at -80°C for future processing.

Melanoma 96-gene panel expression analysis for CTCs: The pre-amplified cDNA was subjected to qPCR to determine expression patterns of target 96 genes, “Melanoma CTC gene panel”, using TaqMan assays and the Biomark HD instrument. The assay was conducted following the

manufacturer's protocol with optimizations for this study. After processing, Raw C_t values generated by Biomark HD (Fluidigm) were analyzed using the SINGuLAR toolset (Fluidigm, USA) and R script to determine the expression pattern of the panel of 96 genes for each sample.^[51, 52] Undetected transcripts automatically generate a C_t value of 999, which were changed to C_t of 40 for numerical analyses.^[51, 52] Further statistical analysis was performed using R software.

SINGuLAR platform for CTCs: To study the gene expression profile of the melanoma CTCs in patient samples, we chose the SINGuLAR Analysis Toolset. The platform supports the gene expression analysis on the qRT-PCR data from the BiomarkHD system and a panel of 96 genes was selected to understand the variations in gene expression between the patient, healthy samples and melanoma cell line. Gene expression data set for each sample is processed according to the guidelines of the SINGuLAR manual ahead of statistical analysis of the data. The raw mRNA expression data from BiomarkHD was grouped and analyzed in a manner that allows for a thorough comparative study of the single gene expression in CTCs before/after surgery for the same patient. The approach adopted in this study also successfully highlights the general trend of gene expression for CTCs and their key variations from the healthy patient samples and the melanoma cell line (positive control). Statistical tools of ANOVA and principal component analysis (PCA) were employed to identify the most significant out of the 96 genes studied. Heatmap with clustering (based on global z score) helped visualize highly correlated genes groups for samples pre- and post-op based on the similarity of their gene expression pattern. Violin and box plots had been used to closely study the degree of expression of a given significant gene across sample groups and thereby help map their trends.

Statistical Analysis: All results present as mean \pm standard deviation. Statistical analysis was demonstrated using Prism software. Unpaired t-tests (two-tailed) were used to compare the differences between melanoma patients (n = 45) versus healthy controls (n = 9). Statistical significance was defined as a two-tailed $p < 0.05$. Gene expression analysis was conducted using the SINGuLAR Analysis Toolset (Fluidigm), which is operated through R. Statistical tools of ANOVA and principal component analysis (PCA) were employed to identify the most significant markers out of the 96 genes studied.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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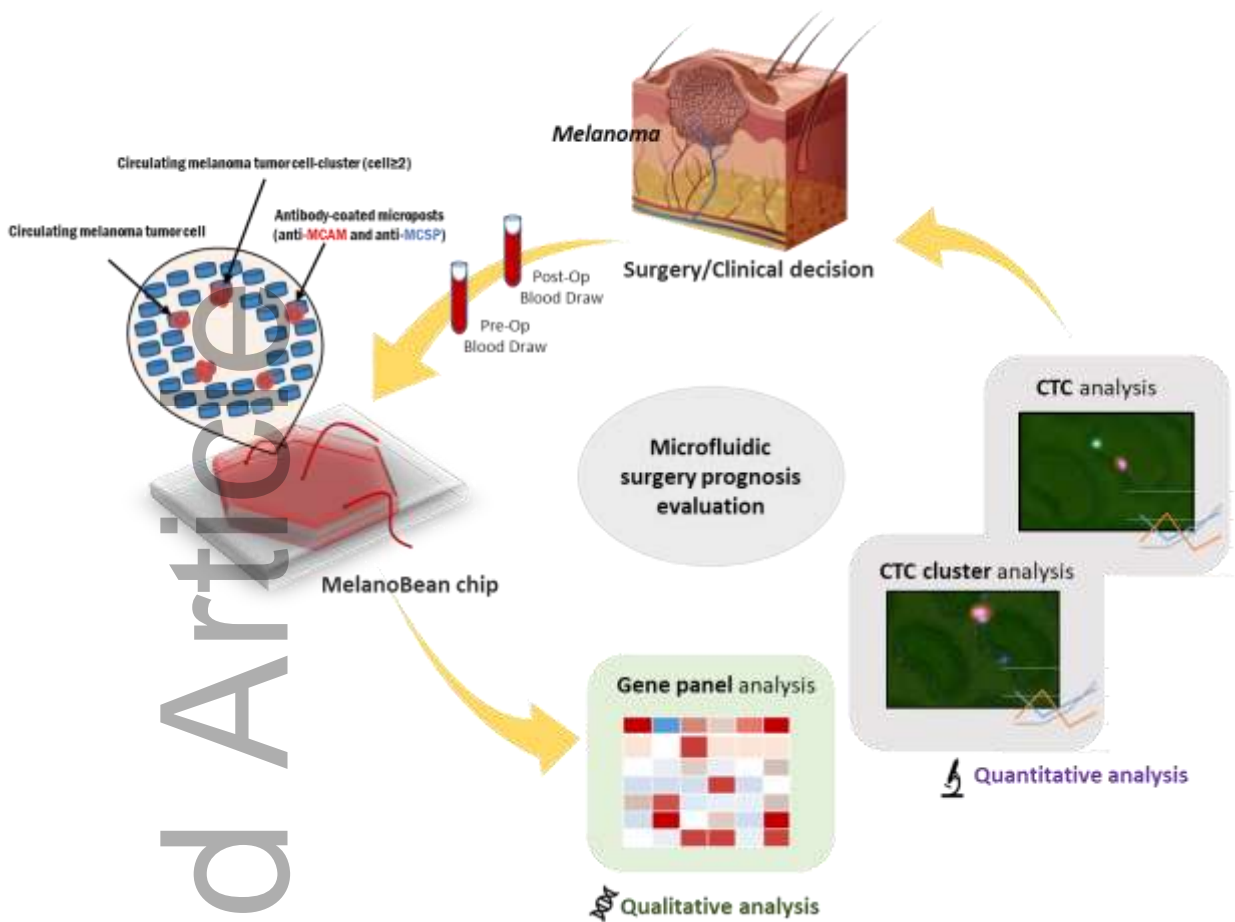


Figure 1. Schematic diagram of the present study for melanoma diagnosis and surgery prognosis evaluation using a microfluidic MelanoBean chip conjugated with anti-MCAM and anti-MCSP antibodies.

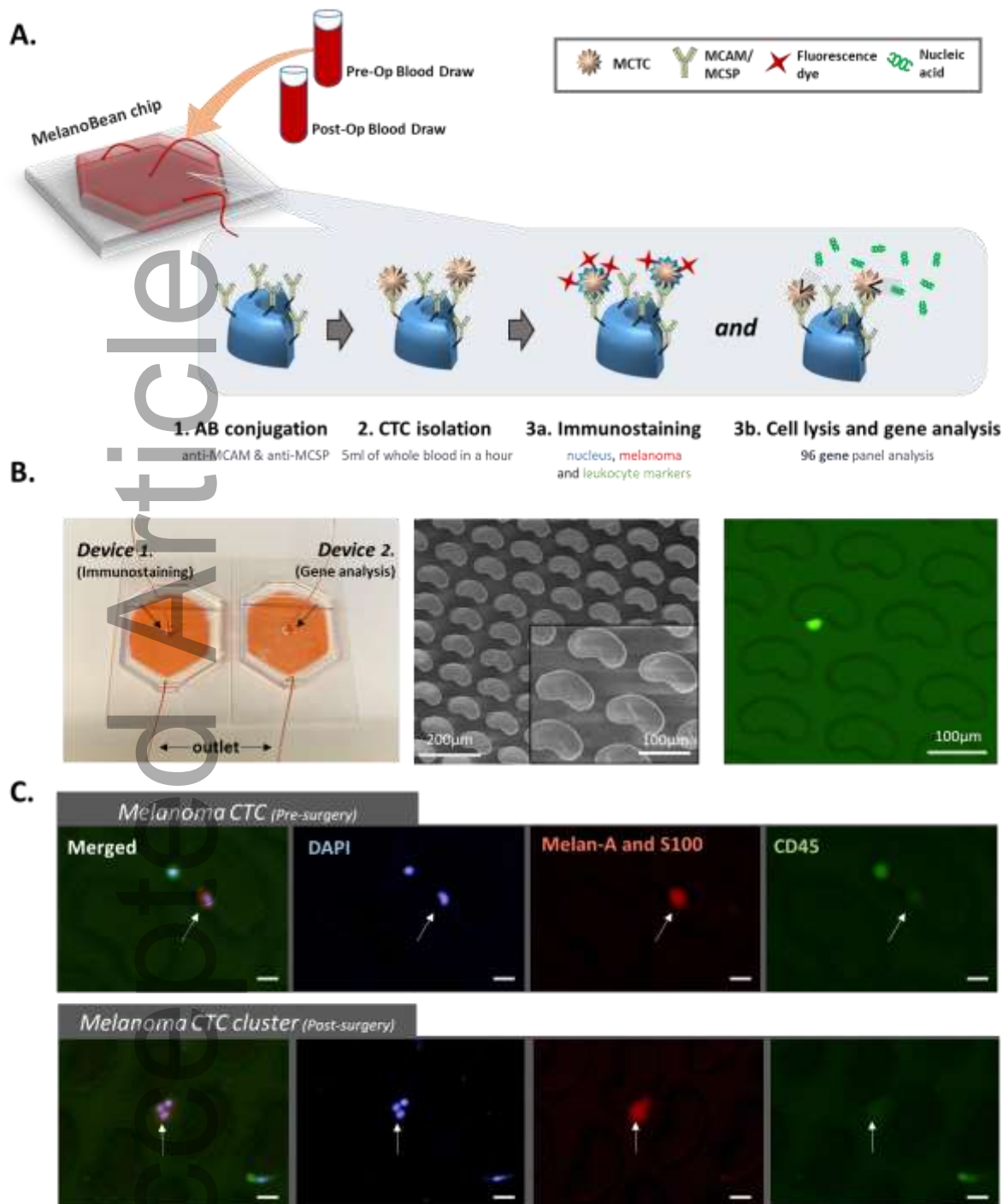


Figure 2. Isolation of melanoma-associated circulating tumor cells and clusters using MelanoBean chip: (A) Procedure of the present study to evaluate pre- and post- surgery efficacy; (B) The fabricated MelanoBean platform (left), enlarged view of bean-shaped microposts using scanning electron microscope (center), and an isolated melanoma cell on the post (right); (C) isolation of melanoma CTC and CTC cluster from melanoma patient samples (All scale bars=20µm)

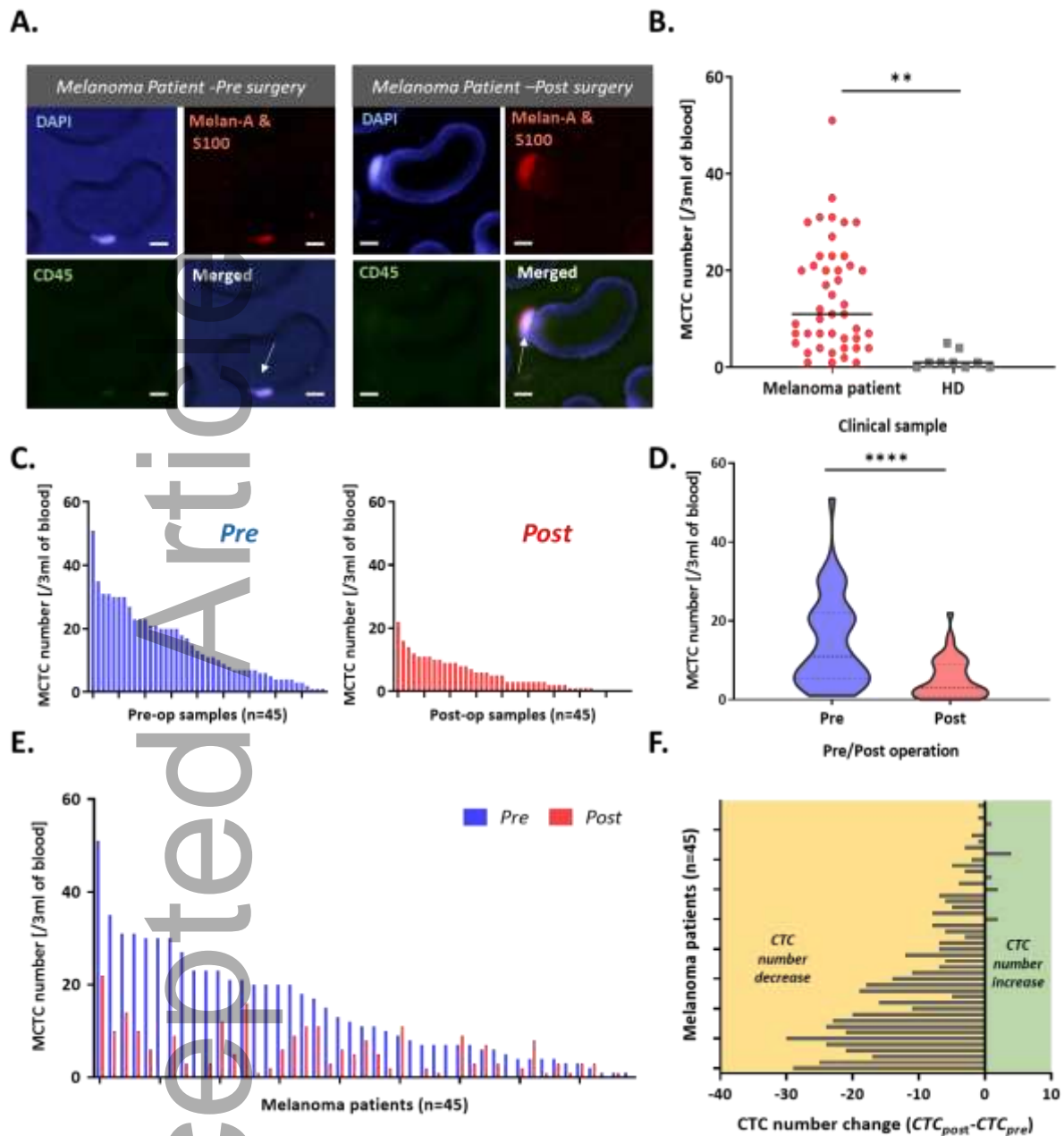


Figure 3. Melanoma circulating tumor cell isolation before/after surgery: (A) Representative images of isolated circulating tumor cells using the MelanoBean platform (All scale bars=20µm) ; (B) MCTC number comparison between melanoma patients (Pre-op, n=45) and healthy donors (n=9) (unpaired t-test); (C) CTC detection frequency comparison between pre- and post- surgery; (D) violin plot of CTC isolated in pre/post-surgery phase (n=45); (E) CTC number changes in paired pre/post-surgery samples; (F) CTC number increase and decrease trends among samples.

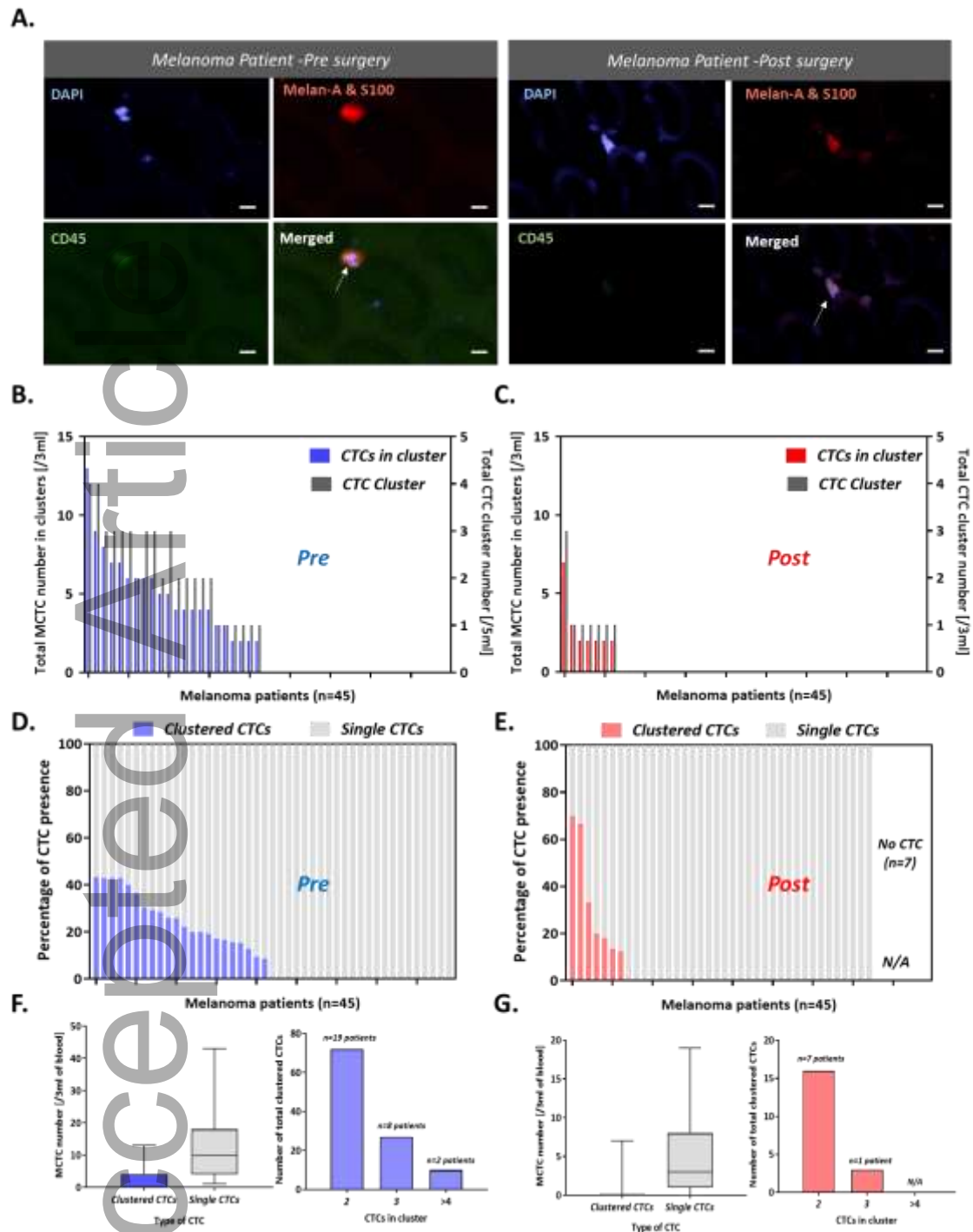


Figure 4. Melanoma circulating tumor cell cluster isolation before/after surgery: (A) Representative images of isolated circulating tumor cell cluster using the MelanoBean platform (Scale bar=20 μ m); (B-C) number of CTCs in clusters and cluster in blood samples at pre-surgery (B) and post-surgery (C); (D-E) portion of clustered CTCs and single CTC at pre (D) and post-surgery (E); (F-G) average number of clustered/single CTCs (left) and number of CTCs per cluster (right) at pre (F) and post-surgery (G)

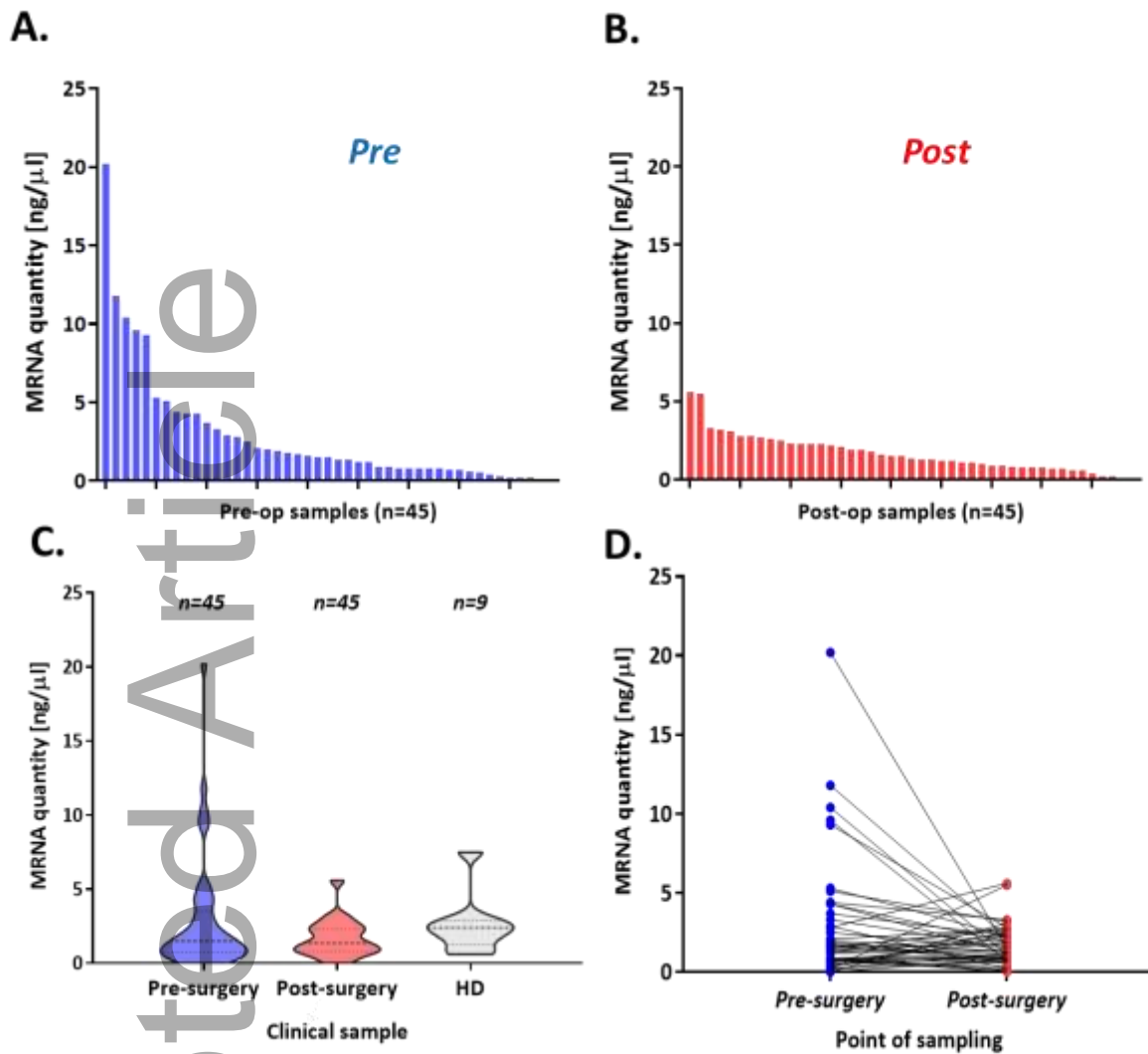


Figure 5. Melanoma RNA quantity comparison before/after surgery: (A-B) RNA quantities of 45 melanoma patients at pre-surgery (A) and post-surgery (B); (C) RNA quantity comparison between melanoma patients at pre/post-surgery and healthy donors; (D) RNA quantity changes before/after surgery

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Figure 6. Gene panel analysis of melanoma CTCs (MCTCs) before/after surgery isolated by MelanoBean chip: **(A)** heatmap analysis of gene expression on MCTCs recovered from melanoma patients before surgery, melanoma cell lines and healthy donors; **(B)** heat map analysis of gene expression on MCTCs recovered from melanoma patients after surgery, melanoma cell lines, and healthy donors; **(C)** pair-wise comparison between MCTCs between pre/post surgery with cell lines and healthy donors.

Table 1. Metastatic melanoma patient characteristics

Characteristics	No. patients [%]	No. of patients w/ CTC [%]	Average CTCs mL ⁻¹
Total patients	45 (100)	45 (100.00)	4.88
Sex			
Male	31 (68.89)	31 (100.00)	4.49
Female	14 (31.11)	14 (100.00)	5.74
Age (35–91)			
<50	6 (13.33)	6 (100)	6.28
≥50	39 (86.67)	39 (100)	4.67
Stage			
I (IA-IB)	21 (46.67)	21 (100)	6.02
II (IIA-IIIC)	13 (28.89)	13 (100)	3.64
III(III, IIIA-IIID)	10 (22.22)	10 (100)	3.43
IV	1 (2.22)	1 (100)	11.67
Surgery			
Wide local excision	43 (95.56)	43 (100)	5.01
Sentinel node biopsy	38 (84.44)	38 (100)	5.28
Complete lymph node dissection	8 (17.78)	8 (100)	3.89

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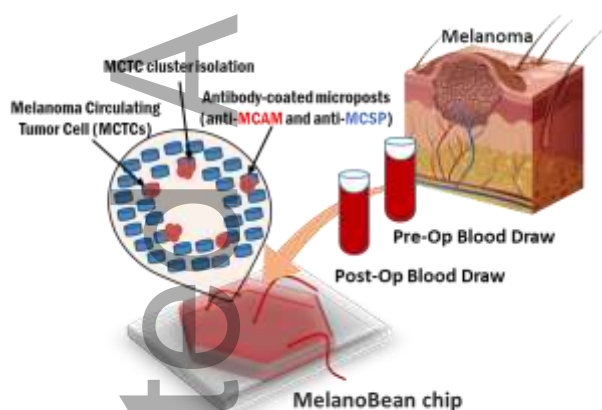
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Table of Contents

Keyword: **Surgical treatment efficacy evaluation using microfluidic CTC isolation**

Yoon-Tae Kang¹, Anusha Mutukuri¹, Thomas Hadlock¹, Heather Fairbairn¹, Taylor R. Carle³, Shamileh Fouladdel¹, Vasudha Murlidhar¹, Aaron Kramer¹, Monica De Silva Reguera¹, Ebrahim Azizi², Alison Durham³, Scott A. Mclean^{3,*} and Sunitha Nagrath^{1,4,*}

Isolation of circulating tumor cells to diagnose melanoma and evaluate the efficacy of surgical resection using melanoma-specific microsystem



Melanoma is one of the most aggressive skin cancers and risk of metastasis is increased with later detection and increased thickness of the primary lesion, thus surgical removal is critical for higher survival rates. Here, we devised microfluidic platform for **diagnosing melanoma and evaluating surgical efficacy** based on change in the number and characteristics of **CTCs/CTC clusters pre- and post-surgical treatment**.