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Isolation of Circulating Tumor Cells to Diagnose Melanoma and Evaluate the Efficacy of Surgical Resection Using Melanoma-Specific Microsystem

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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/surgical removal is critical for higher survival rates. Recent advances in liquid biopsy have proposed less-invasive alternatives for cancer diagnosis and monitoring using minimal invasion at sample collection, and circulating tumor cells (CTCs) have been considered a promising blood-based surrogate marker of primary tumors. Herein, the melanoma-specific OncoBean platform (MelanoBean) conjugated with melanoma specific antibodies is used. From the comprehensive studies based on change in the number and characteristics of CTCs/CTCs-clusters pre- and post-surgical treatment, this work demonstrates that melanoma patients ($n = 45$) at all stages (I–IV) have a noticeable number of CTCs as well as CTC-clusters compared to healthy donors ($n = 9$) ($p = 0.0011$), and surgical treatment leads to a significant decrease in the CTC number ($p < 0.0001$). The CTCs recovered from the device undergo 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 a 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]

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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 mL^{-1} 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 offer 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] Semiautomated 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 toward 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 (**Figure 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 MB Microfluidic Device

MB chips were fabricated using a standard soft-lithography method following previously defined procedures[40] and functionalized with antibodies against MCAM and MCSP (**Figure 2**A). 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 (Figure 2B, left). To confirm the ability of the MB 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 prestained 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 200–1000 cells mL^{-1} 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

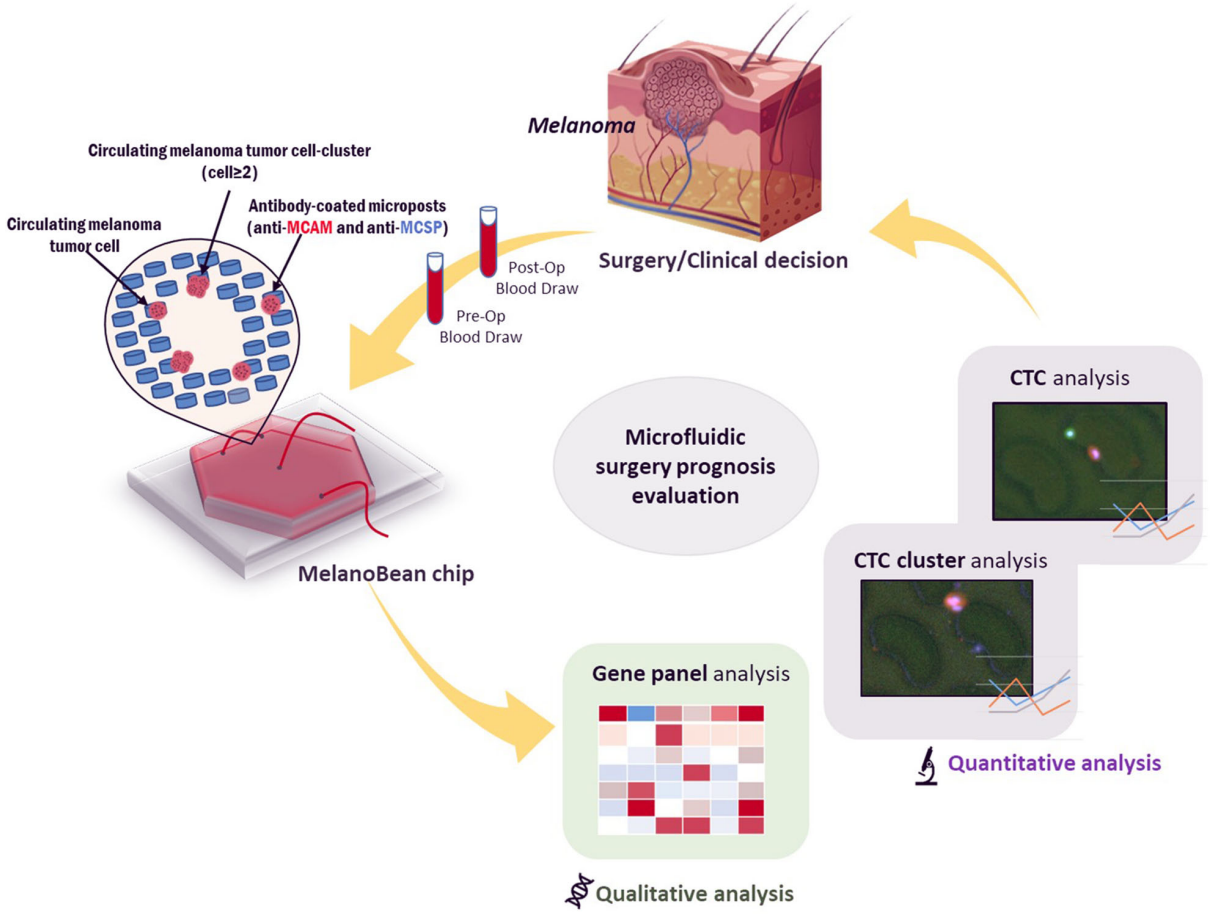

Figure 1. Schematic diagram of the present study for melanoma diagnosis and surgery prognosis evaluation using a microfluidic MB chip conjugated with anti-MCAM and anti-MCSP antibodies.

before and after surgical resection were applied to separate two MCAM/MCSP functionalized MB devices.

One presurgery and one postsurgery device were fluorescently stained for enumeration, while the second processed pre- and postsurgery 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 MB devices successfully isolate MCTCs on the surface of the PDMS bean-shaped posts. Fluorescent images in Figure 2C confirm that 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 the 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 h 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 3**A). Selection criteria for positive identification of MCTCs include shape, size, and fluorescence (DAPI+/CY3+/FITC–). In total, 45 melanoma patients (**Table 1**) were examined before and after surgery, along with

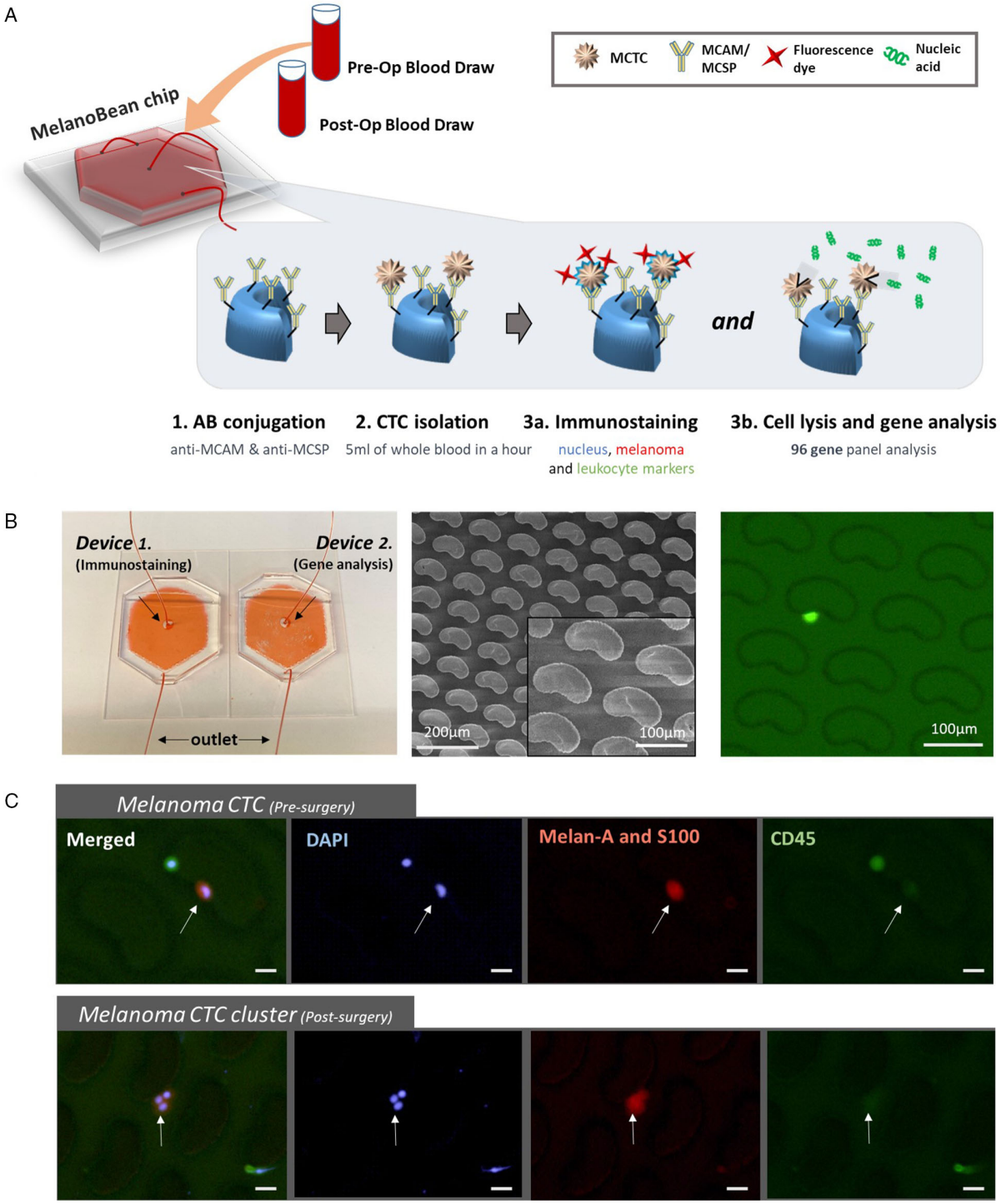

Figure 2. Isolation of melanoma-associated circulating tumor cells and clusters using MB chip: A) procedure of the present study to evaluate pre- and postsurgery efficacy; B) the fabricated MB platform (left), enlarged view of bean-shaped microposts using scanning electron microscope (center), and an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

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 1–5/3 mL. Average MCTC counts in patient samples (pre-op) were significantly higher, 14.64 ± 11.35 MCTCs/3 mL blood while healthy donors averaged just 1.44 ± 1.81 MCTCs/3 mL blood ($p = 0.0011$) (Figure 3B). Next, we compared the MCTC numbers isolated from patients at pre- and post-op

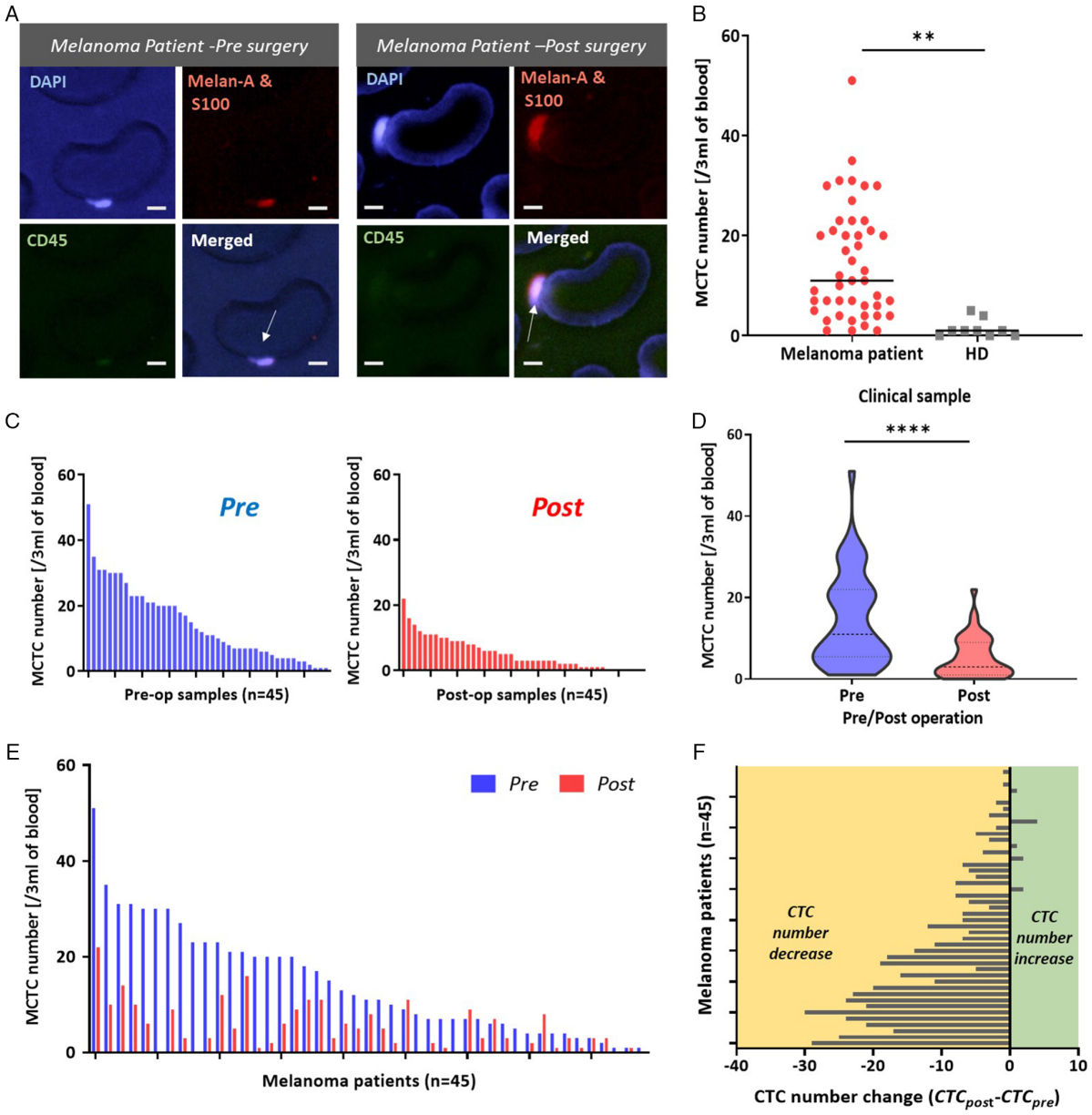

Figure 3. MCTC isolation before/after surgery: A) representative images of isolated circulating tumor cells using the MB platform (all scale bars = 20 μm); B) MCTC number comparison between melanoma patients (pre-op, $n = 45$) and healthy donors ($n = 9$) (unpaired t-test); C) CTC detection frequency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

(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 to 51 CTCs (average = 14.64) ($p < 0.0001$, 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 ($p < 0.0001$, paired t-test). We next evaluated whether changes in the MCTC number after surgery are predictive of patient response to surgery (Figure S1, Supporting Information). 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 seven patients who did not show a reduction in MCTCs, two had no change, while five 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

Table 1. Metastatic melanoma patient characteristics.

Characteristics	No. of patients [%]	No. of patients with 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–IIC)	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

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 min.[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 h 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.

2.3. MCTC Cluster Counts Before and After Surgery

The presence and clinical implications of CTC clusters in melanoma and clinical implications have 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 Figure 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 nine healthy donor samples had clusters.

The average numbers in MCTCs found in clusters were 5.05 and 2.86 at pre- 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 4). 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 to 6 MCTCs, while those in either post-op group ranged from 2 to 3 MCTCs (Figure 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 ($p < 0.0001$), 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 are 0.44 and 4.82 ($p < 0.0001$), respectively. It is also noteworthy that of these clusters, more than 66% of MCTCs found in clusters are in clusters containing only two MCTCs in both pre- and postcases even though various sizes of MCTC clusters have been reported previously. Further studies are needed for a clearer conclusion.

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 **Figure 5**A,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- and postsurgery 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 yield this variance.

As shown in Figure 5, 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 nonspecific cells, such as leukocytes,

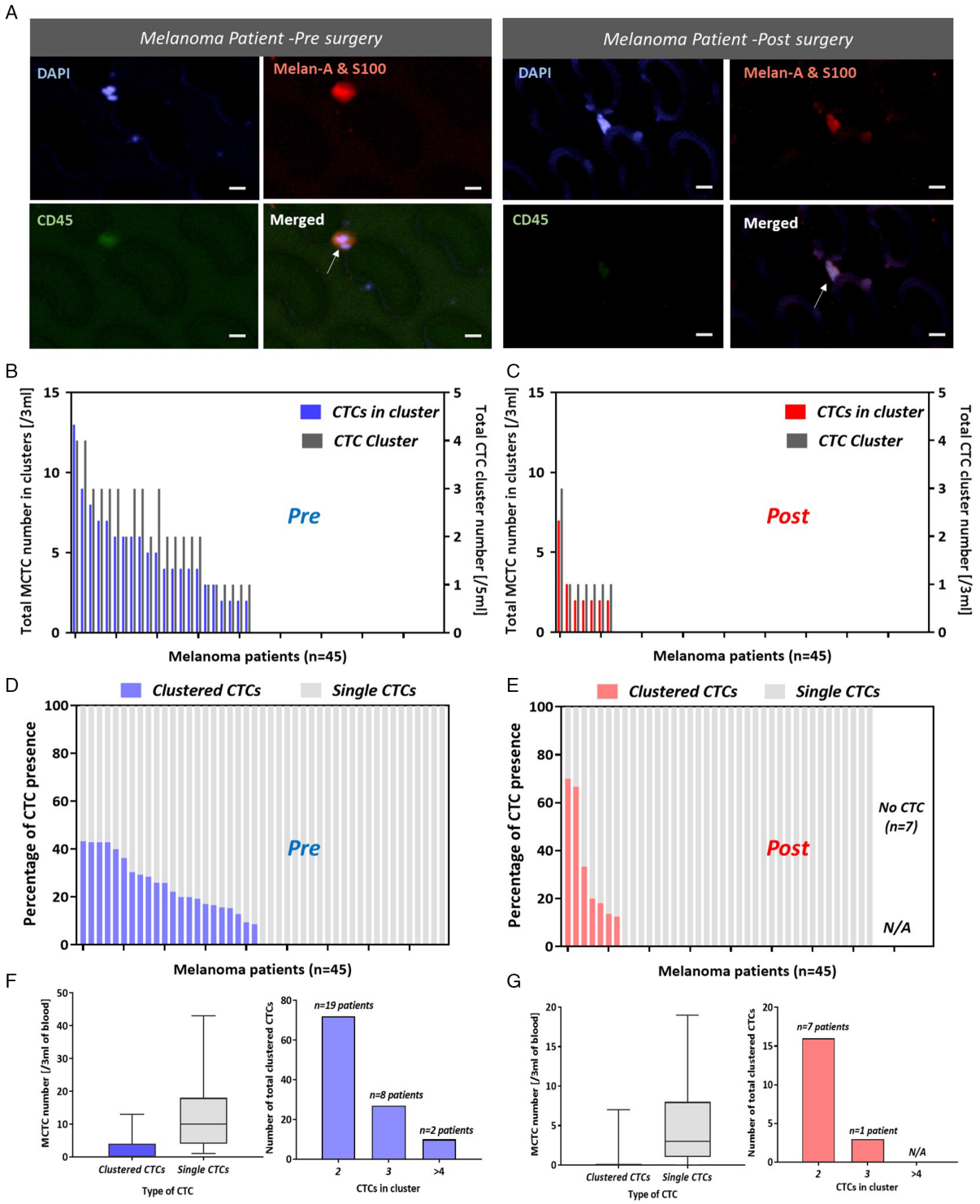

Figure 4. MCTC cluster isolation before/after surgery: A) representative images of isolated circulating tumor cell cluster using the MB platform (scale bar = 20 μm); B,C) number of CTCs in clusters and cluster in blood samples at presurgery (B) and postsurgery (C); D,E) portion of clustered CTCs and single CTC at presurgery (D) and postsurgery (E); F,G) average number of clustered/single CTCs (left) and number of CTCs per cluster (right) at presurgery (F) and postsurgery (G).

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.

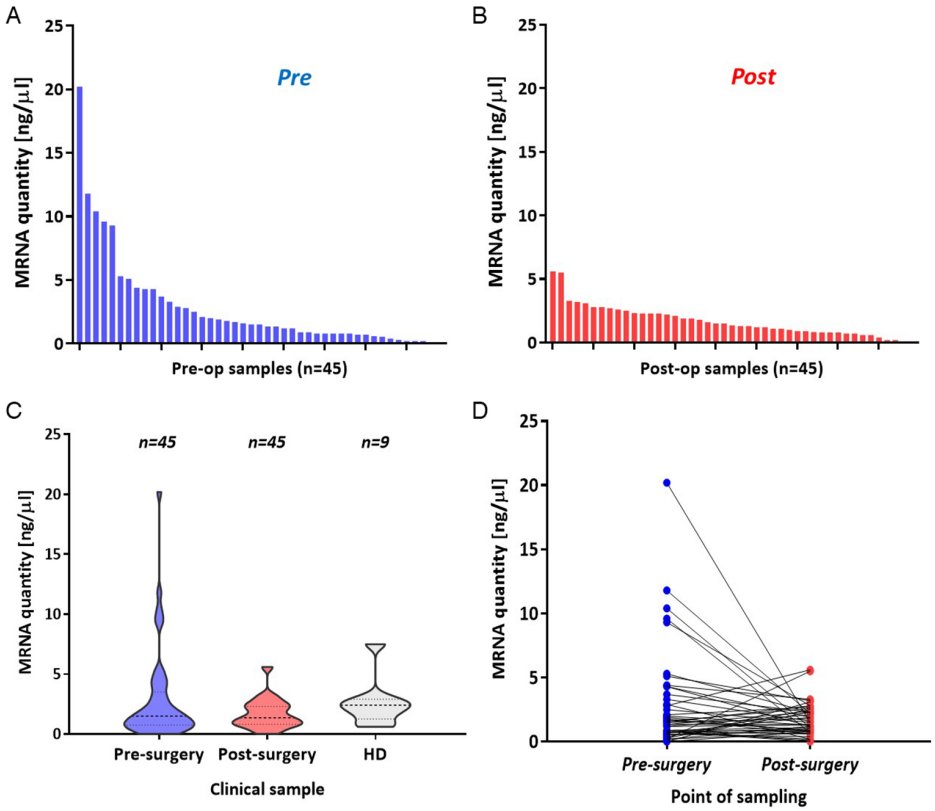

Figure 5. Melanoma RNA quantity comparison before/after surgery: A,B) RNA quantities of 45 melanoma patients at presurgery (A) and postsurgery (B); C) RNA quantity comparison between melanoma patients at pre-/postsurgery and healthy donors; D) RNA quantity changes before/after surgery.

2.5. 96 Gene Expression Profiling of Captured MCTCs

Following isolation of MCTCs by MB 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 postoperative gene expression results for each patient are displayed in **Figure 6**A,B. As we see from these results, several genes are highly expressed among most samples tested (Figure 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 among 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 among the profiled genes, some samples showed significant uniformity in their overall expression. In particular, several samples showed little to no expression among 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 postsurgery samples (Figure 6B).

The generated 96 gene profiles from each patient's pre- and postoperation samples were then compared, as shown in Figure 6C. As shown by this heatmap, several genes had a notable change in expression following surgery among the cohort. First off, while CD44 appears to show significant expression among most tested samples, a decrease in its expression can be found in 16 of the 27 patients following surgery. CD44 gene and its isoform have been known to have correlations with tumor growth and metastasis, thus have 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 decreased following surgery. LIF, which in general showed very low expression among samples, demonstrated a slight increase in

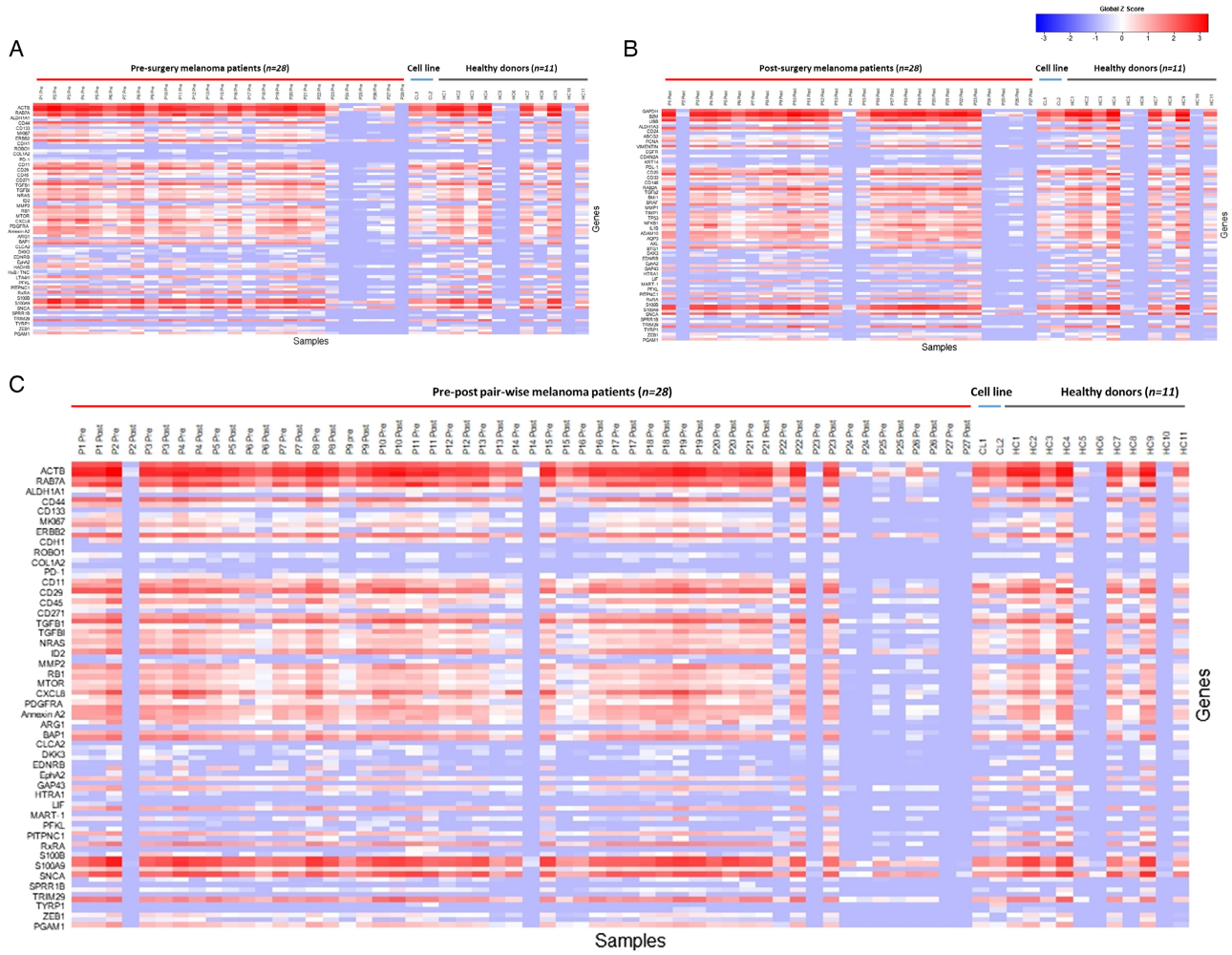

Figure 6. Gene panel analysis of melanoma CTCs (MCTCs) before/after surgery isolated by MB chip: A) heatmap analysis of gene expression on MCTCs recovered from melanoma patients before surgery, melanoma cell lines, and healthy donors; B) heat map analysis of gene expression on MCTCs recovered from melanoma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

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 among almost all the genes tested in their profiles to others. Even among the genes that trended toward 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.

3. 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 1 mL of whole blood, while healthy donors have less than 0.47 MCTCs mL^{-1}. 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.

4. Experimental Section

Melanoma Cell Culture and Model Sample Preparation: SK-MEL-103 cell line was used for the preparation of our model sample. SK-MEL-103 cells were cultured in conditioned media, followed by spiking of 5000 cells 1 mL of whole blood sample. Cells were prestained by CellTracker dye (green) and isolated cells were evaluated using fluorescence microscopy.

Clinical Sample Collection and Preparation: All experiments and sample collection were 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 6 mL 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 half an hour of tumor removal. All samples were collected into K3 EDTA blood collection tubes (BD, USA) and CTC isolation was performed within 2 h of blood collection.

MB 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, O_2 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 min. A 500 µL silane in 5 mL 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 µL GMBS in 5 mL ethanol) which was allowed to incubate for 30 min before another ethanol wash. The injection of a 500 µL NeutrAvidin in 5 mL PBS solution was the final step before each device was 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, $IgG1_K$, anti-human, Miltenyi Biotec, Germany) and MCSP (Mouse host, $IgG1_K$, anti-human, Novus, USA). A 250 µL antibody solution containing 2.5 µL of anti-MCAM (2.5 µg, 1 mg mL^{-1}) and 2.76 µL anti-MCSP (2.5 µg, 0.91 mg mL^{-1}) with the remainder 1% BSA was injected into each device and allowed to incubate for 1 h. 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 wash, 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 3 mL of whole blood into each antibody-conjugated device, MB chip, at a flow rate of 5 mL h^{-1}. Blood remaining in the devices was then immediately washed out using PBS at a flow rate of 10 mL h^{-1}. 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 1 mL of 4% PFA solution (250 µL of 16% PFA in 750 µL PBS). PFA fixation solution was allowed to incubate for 40 min before being washed out with PBS. Each device was then permeabilized with 1 mL of 0.2% Triton solution and incubated 30 min. Triton was then removed by PBS wash before the application of 1 mL of 3% BSA–2% normal goat serum solution (500 µL of 6% BSA, 200 µL normal goat serum, 300 µL PBS) which was incubated for 30 min. Primary staining antibody solution composed of 10 µL anti-Melan-A/MART1 (R&D Systems, USA), 25 µL S100 (mouse IgG2a, ThermoFischer, USA), and 25 µL CD45 (rat IgG2b, Santa Cruz Biotech, USA) in 1 mL of 1% BSA was pumped into each device and incubated for 1 h. Excess primary antibody solution was removed by PBS wash. A secondary staining antibody solution was then applied containing 5 µL AlexaFluor 546 (goat anti-mouse IgG2a, Life Technologies, USA) and 5 µL AlexaFluor 488 (goat anti-rat IgG, Life Technologies, USA) in 1 mL of 1% BSA and allowed to incubate for 1 h in the dark. Excess secondary antibodies were removed by PBS wash followed by the application of 1 mL DAPI staining solution (1 µL DAPI in 1 mL of 1% BSA.) The DAPI solution was incubated for 15 min followed by a final PBS wash. Devices are imaged using Ti2 microscope (Nikon, Japan) at 10× magnification for cell analysis. Images were taken in FITC, DAPI, and CY3 fluorescence.

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

Melanoma 96-Gene Panel Expression Analysis for CTCs: The preamplified 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 was 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 ± 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-tailored $p < 0.05$. Gene expression analysis was conducted using the SINGuLAR Analysis Toolset (Fluidigm), which is operated through R. Statistical tools of ANOVA and 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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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Keywords

circulating tumor cells, liquid biopsy, melanoma, microsystem, surgical treatment efficacy evaluation

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[1] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, A. Jemal, *Ca Cancer J. Clin.* **2018**, *68*, 394.
[2] E. Maverakis, L. A. Cornelius, G. M. Bowen, T. Phan, F. B. Patel, S. Fitzmaurice, Y. He, B. Burrall, C. Duong, A. M. Kloxin, H. Sultani, R. Wilken, S. R. Martinez, F. Patel, *Acta. Derm. Venereol.* **2015**, *95*, 516.
[3] L. E. Davis, S. C. Shalin, A. J. Tackett, *Cancer Biol. Ther.* **2019**, *20*, 1366.
[4] D. Okwan-Duodu, B. P. Pollack, D. Lawson, M. K. Khan, *Am. J. Clin. Oncol.* **2015**, *38*, 119.
[5] R. Younes, F. C. Abrao, J. Gross, *Melanoma Res.* **2013**, *23*, 307.
[6] J. Wong, K. Skinner, K. Kim, L. Foshag, D. Morton, *Surgery* **1993**, *113*, 389.
[7] J. A. Sosman, J. Moon, R. J. Tuthill, J. A. Warneke, J. T. Vetto, B. G. Redman, *Cancer* **2011**, *117*, 4740.
[8] S. K. Huang, D. S. B. Hoon, *Mol. Oncol.* **2016**, *10*, 450.
[9] S. B. Lim, W. D. Lee, J. Vasudevan, W. T. Lim, C. T. Lim, *NPJ Precis. Oncol.* **2019**, *3*, 23.
[10] W. Zhang, W. Xia, Z. Lv, Y. Xin, C. Ni, L. Yang, *Cell. Physiol. Biochem.* **2017**, *41*, 755.
[11] E. Kidess, S. S. Jeffrey, *Genome Med.* **2013**, *5*, 70.
[12] B. Rack, C. Schindlbeck, J. Jückstock, U. Andergassen, P. Hepp, T. Zwingers, T. W. P. Friedl, R. Lorenz, H. Tesch, P. A. Fasching, T. Fehm, A. Schneeweiss, W. Lichtenegger, M. W. Beckmann, K. Friese, K. Pantel, W. Janni, *J. Natl. Cancer Inst.* **2014**, *106*, djc273.
[13] M. Kim, D. H. Suh, J. Y. Choi, J. Bu, Y. T. Kang, K. Kim, J. H. No, Y. B. Kim, Y. H. Cho, *Medicine* **2019**, *98*, e15354.
[14] C. Alix-Panabières, K. Pantel, *Nat. Rev. Cancer* **2014**, *14*, 623.
[15] G. Vona, G. Sabile, M. Louha, V. Sitruk, S. Romana, K. Schütze, F. Capron, D. Franco, M. Pazzagli, M. Vekemans, B. Lacour, C. Bréchot, P. Paterlini-Bréchot, *Am. J. Pathol.* **2000**, *156*, 57.
[16] Y. T. Kang, I. Doh, J. Byun, H. J. Chang, Y. H. Cho, *Theranostics* **2017**, *7*, 3179.
[17] S. Zheng, H. Lin, J. Q. Liu, M. Balic, R. Datar, R. J. Cote, Y. C. Tai, *J. Chromatogr., A* **2007**, *1162*, 154.
[18] R. Gertler, R. Rosenberg, K. Fuehrer, M. Dahm, H. Nekarda, J. R. Siewert, *Recent Results Cancer Res.* **2003**, *162*, 149.
[19] J. Weitz, P. Kienle, J. Lacroix, F. Willeke, A. Benner, T. Lehnert, C. Herfarth, M. Von Knebel Doeberitz, *Clin. Cancer Res.* **1998**, *4*, 343.
[20] Y.-T. Kang, Y. J. Kim, B. Rupp, E. Purcell, T. Hadlock, N. Ramnath, S. Nagrath, *Anal. Sens.* **2021**, *1*, 117.
[21] E. I. Galanzha, E. V. Shashkov, P. M. Spring, J. Y. Seun, V. P. Zharov, *Cancer Res.* **2009**, *69*, 7926.
[22] S. Nagrath, L. V. Sequist, S. Maheswaran, D. W. Bell, D. Irimia, L. Ulkus, M. R. Smith, E. L. Kwak, S. Digumarthy, A. Muzikansky, P. Ryan, U. J. Balis, R. G. Tompkins, D. A. Haber, M. Toner, *Nature* **2007**, *450*, 1235.
[23] R. M. Mohamadi, J. D. Besant, A. Mepham, B. Green, L. Mahmoudian, T. Gibbs, I. Ivanov, A. Malvea, J. Stojcic, A. L. Allan, L. E. Lowes, E. H. Sargent, R. K. Nam, S. O. Kelley, *Angew. Chem., Int. Ed.* **2015**, *54*, 139.
[24] J. Zhou, A. Kulasinghe, A. Bogseth, K. O'Byrne, C. Punyadeera, I. Paautsky, *Microsyst. Nanoeng.* **2019**, *5*, 8.
[25] H. J. Yoon, T. H. Kim, Z. Zhang, E. Azizi, T. M. Pham, C. Paoletti, J. Lin, N. Ramnath, M. S. Wicha, D. F. Hayes, D. M. Simeone, S. Nagrath, *Nat. Nanotechnol.* **2013**, *8*, 735.
[26] S. Wang, H. Wang, J. Jiao, K. J. Chen, G. E. Owens, K. I. Kamei, J. Sun, D. J. Sherman, C. P. Behrenbruch, H. Wu, H. R. Tseng, *Angew. Chem., Int. Ed.* **2009**, *48*, 8970.
[27] H. Cho, J. Kim, H. Song, K. Y. Sohn, M. Jeon, K.-H. Han, *Analyst* **2018**, *143*, 2936.
[28] V. D. Giorgi, P. Pinzani, F. Salvianti, J. Panelo, M. Paglierani, A. Janowska, M. Grazzini, J. Wechsler, C. Orlando, M. Santucci, T. Lotti, M. Pazzagli, D. Massi, *J. Invest. Dermatol.* **2010**, *130*, 2440.
[29] A. Lucci, C. Hall, S. P. Patel, B. Narendran, J. B. Bauldry, R. Royal, M. Karhade, J. R. Upshaw, J. A. Wargo, I. C. Glitza, M. K. K. Wong, R. N. Amaria, H. A. Tawbi, A. Diab, M. A. Davies, J. E. Gershenwald, J. E. Lee, P. Hwu, M. I. Ross, *Clin. Cancer Res.* **2020**, *26*, 1886.
[30] C. Rao, T. Bui, M. Connelly, G. Doyle, I. Karydis, M. R. Middleton, G. Clack, M. Malone, F. A. Coumans, L. W. Terstappen, *Int. J. Oncol.* **2011**, *38*, 755.
[31] L. Khoja, P. Lorigan, C. Zhou, M. Lancashire, J. Booth, J. Cummings, R. Califano, G. Clack, A. Hughes, C. Dive, *J. Invest. Dermatol.* **2013**, *133*, 1582.
[32] C. S. Hall, M. Ross, J. B. B. Bauldry, J. Upshaw, M. G. Karhade, R. Royal, S. Patel, A. Lucci, *J. Am. Coll. Surg.* **2018**, *227*, 116.
[33] J. Y. Pierga, F. C. Bidard, C. Mathiot, E. Brain, S. Delaloge, S. Giachetti, P. D. Cremoux, R. Salmon, A. Vincent-Salomon, M. Marty, *Clin. Cancer Res.* **2008**, *14*, 7004.
[34] J. R. Marshall, M. R. King, *Transl. Cancer Res.* **2016**, *5*, S126.
[35] G. Marsavela, C. A. Aya-Bonilla, M. E. Warkiani, E. S. Gray, M. Ziman, *Cancer Lett.* **2018**, *424*, 1.
[36] D. Klinac, E. S. Gray, J. B. Freeman, A. Reid, S. Bowyer, M. Millward, M. Ziman, *BMC Cancer* **2014**, *14*, 423.

[37] X. Hong, R. J. Sullivan, M. Kalinich, T. T. Kwan, A. Giobbie-Hurder, S. Pan, J. A. LiCausi, J. D. Milner, L. T. Nieman, B. S. Wittner, U. Ho, T. Chen, R. Kaur, D. P. Lawrence, K. T. Flaherty, L. V. Sequist, S. Ramaswamy, D. T. Miyamoto, M. Lawrence, M. Toner, *PNAS* **2018**, *115*, 2467.
[38] V. Murlidhar, M. Zeinali, S. Grabauskiene, M. Ghannad-Rezaie, M. S. Wicha, D. M. Simeone, N. Ramnath, R. M. Reddy, S. Nagrath, *Small* **2014**, *10*, 4895.
[39] V. Murlidhar, R. M. Reddy, S. Fouladdel, L. Zhao, M. K. Ishikawa, S. Grabauskiene, Z. Zhang, J. Lin, A. C. Chang, P. Carrott, W. R. Lynch, M. B. Orringer, C. Kumar-Sinha, N. Palanisamy, D. G. Beer, M. S. Wicha, N. Ramnath, E. Azizi, S. Nagrath, *Cancer Res.* **2017**, *77*, 5194.
[40] Y. T. Kang, T. Hadlock, T. W. Lo, E. Purcell, A. Mutukuri, S. Fouladdel, M. D. S. Raguera, H. Fairbairn, V. Murlidhar, A. Durham, S. A. McLean, S. Nagrath, *Adv. Sci.* **2020**, *7*, 2001581.
[41] M. A. Juratli, M. Sarimollaoglu, E. R. Siegel, D. A. Nedosekinl, E. I. Galanzha, J. Y. Suend, V. P. Zharov, *Head Neck* **2014**, *36*, 1207.
[42] P. Quaglino, S. Osella-Abate, N. Cappello, M. Ortoncelli, T. Nardò, M. T. Fierro, F. Cavallo, P. Savoia, M. G. Bernengo, *Melanoma Res.* **2007**, *17*, 75.
[43] L. R. Jiao, C. Apostolopoulos, J. Jacob, R. Szydlo, N. Johnson, J. Tsim, N. A. Habib, R. C. Coombes, J. Stebbing, *J. Clin. Oncol.* **2009**, *27*, 6160.
[44] E. I. Galanzha, V. P. Zharov, *Cancers* **2013**, *5*, 1691.
[45] E. I. Galanzha, Y. A. Menyaev, A. C. Yadem, M. Sarimollaoglu, M. A. Juratli, D. A. Nedosekin, S. R. Foster, A. Jamshidi-Parsian, E. R. Siegel, I. Makhoul, L. F. Hutchins, J. Y. Suen, V. P. Zharov, *Sci. Transl. Med.* **2019**, *11*, eaat5857.
[46] P. Hai, Y. Qu, Y. Li, L. Zhu, L. Shmuylovich, L. A. Cornelius, L. V. Wang, J. Biomed, *Opt.* **2020**, *25*, 036002.
[47] N. Aceto, A. Bardia, D. T. Miyamoto, M. C. Donaldson, B. S. Wittner, J. A. Spencer, M. Yu, A. Pely, A. Engstrom, H. Zhu, B. W. Brannigan, R. Kapur, S. L. Stott, T. Shioda, S. Ramaswamy, D. T. Ting, C. P. Lin, M. Toner, D. A. Haber, S. Maheswaran, *Cell* **2014**, *158*, 1110.
[48] Y. Zhang, J. Li, L. Wang, P. Meng, J. Zhao, P. Han, J. Xia, J. Xu, L. Wang, F. Shen, S. Zheng, F. Zhou, R. Fan, *Mol. Med. Rep.* **2019**, *20*, 1551.
[49] C. Chen, S. Zhao, A. Karnad, J. W. Freeman, *J. Hematol. Oncol.* **2018**, *11*, 64.
[50] A. Satelli, S. Li, *Cell. Mol. Life Sci.* **2011**, *68*, 3033.
[51] T. D. Schmittgen, K. J. Livak, *Nat. Protoc.* **2008**, *3*, 1101.
[52] K. J. Livak, T. D. Schmittgen, *Methods* **2001**, *25*, 402.