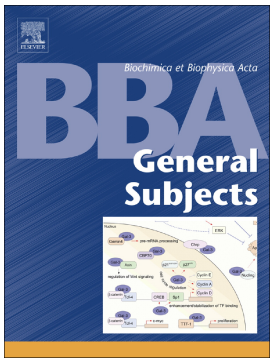

Detection of CD133-marked cancer stem cells by surface plasmon resonance: Its application in leukemia patients

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Detection of CD133-marked cancer stem cells by surface plasmon resonance: its application in leukemia patients

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Abstract

Here, we reported the development of a label-free and real-time surface plasmon resonance (SPR) based biosensor for cancer stem cells (CSCs) detection using cell surface biomarker; CD133. The fabricated biosensor was used for detection of this marker in some acute myeloid leukemia (AML) patients and the results were compared with those obtained from flow cytometry (FC) method. CD133 antibody was immobilized on the gold chip surface via EDC/NHS coupling method and binding of the candidate cells to the modified gold sensor surface was monitored after isolation of mononuclear cells from bone marrow of the patients. The method was validated in terms of various parameters such as CD133-antibody concentration and cell density. The CD133-marked cells were investigated in seven AML patients. All SPR results were compared with those obtained from FC method. A very good correlation (R^2= 0.96) was obtained between SPR and FC responses related to CD133-marked cells densities. In conclusion, in this study, a label-free and real-time SPR cytometry method was developed to detect CD133 and it was successfully applied to follow this cancer stem cell biomarker in AML patients.

Keywords: Cancer stem cells; CD133; Acute myeloid leukemia; SPR; Flow cytometry

1. Introduction

The significance of cancer stem cells (CSCs) is important in the identification and prediction of tumors dynamic. In line with this statement, designing new approaches for the anti-cancer drug targeting CSCs is obvious to all. These cells exhibit more resistance to chemotherapy agents than usual tumor cells, thereby the need for developing new detection methods for CSCs has been enhanced [1, 2]. Recent studies showed that CSCs ,which having the nature of usual stem cells, are responsible for tumor initiation, progression, resistance to chemotherapy and metastasis [3, 4].The presence of CSCs was first confirmed in the context of acute myeloid leukemia (AML) [5]. AML is considered as the most common type of cancer in adults which involve the bone marrow and blood and proved by the emergence of abnormal myeloblasts, red blood cells, and/or platelets. In 1997, Yin for the first time discovered hematopoietic stem cells expressing CD133 (or AC133) originated from the bone marrow [6].

Human CD133, as a member of the prominin family, is a unique five-transmembrane protein [7]. This molecule possesses a pentaspan transmembrane glycoprotein with 865 amino acids and molecular weight of 120 kDa which is highly expressed in hematopoietic stem cells [6]. As shown in **Fig 1a,** structural studies revealed three extracellular domains with large loops at the position of 20-108, 179-433 and 508-592 with the length of 89, 255 and 285 amino acids, respectively. The cytoplasmic domain has 112 amino acids with the C-terminal cytoplasmic tail [8]. Due to having large loops in the extracellular domain, CD133 is suitable for detection using cell surface analyses. Human CD133 antigen was identified in cases of AML, especially myelomonocytic AML from M0 to M5 types [9]. The discovery of CD133 as a most important marker for CSCs was found through different researches. Compared to CD133 negative cells, $CD133^{+}$ counterparts are able to produce a whole mass tumor, confirming multipotentiality properties [10]. As a matter of fact, monitoring the population, and growth dynamics of $CD133^{+}$ cells in the bone marrow or peripheral blood samples could yield improved clinical outcomes. The conventional assays used to find cell reactions are laborious and tedious with several steps such as labeling, washing steps. Consequently, there is a crucial need for label-free cell identification techniques without these limitations. Due to having non-destructive nature and real-time observation of cellular process in label-free SPR-based method, this approach has been applied for the signal measurement in biomedical and pharmaceutical studies [11-14].

Development of SPR sensing methods describes important and precise information about bio-molecular interactions [15, 16]. The application of SPR biosensors have been

focused on the investigation of interactions between protein-peptide, nucleotides, and drug-albumin and also for the probe of cell surface marker-antibody and cellular morphological changes induced by various factors [17-19]. Recently, the application of SPR-based method as optical biosensors was expanded in the field of cells analyses [20-24]. SPR cytometry indicates the assessment of parameters from intact cells using the SPR event [25]. Given that SPR having the penetration depth of plasmonic wave around 400 nm in the gold surface, the use of this method is limited for monitoring micrometer-sized particles, notably cells. However, qualitative and cell surface analysis can still be carried out by researchers [26-29]. For example SPR sensing method has been applied to detect individual hybridoma cells using EpCAM protein immobilized on SPR chips [30]. Accordingly the designing of validated SPR based method in cellular studies would be grate of value and the scope of application this method becomes wider. On the development of SPR-based methods for cell biology studies, conducted by our group, SPR-based method was developed for detection of biomolecule interactions with cells and monitoring cell integrity loaded on gold chips [31-34].

In line with our previous studies as mentioned above, in this study, an effective technique for CSC identification was developed in a label-free and real-time manner. To assess CSCs through an optic-based SPR biosensor, CD133 was targeted in cells from patients with diagnosed AML. Flow cytometric assessment was performed to detect CD133 expression on mononuclear cells (MNCs). The target cells were captured on surfaces coated with an antibody targeting factor CD133. Also, we investigated whether the SPR signal depended on cell surface antigen (CD133) density and explored the suitability of SPR for probing CD133 positive cells in comparison with flow cytometry (FC) analyses. Further, the light microscopic examination of samples stained with Giemsa was investigated in AML subjects.

2. Materials and Methods

2.1. Materials

Ficoll®-Paque PREMIUM 1.073, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride ($C_8H_{17}N_3$.HCl) (EDC), N-hydroxysuccinimide ($C_4H_5NO_3$) (NHS), 11-mercaptoundecanoic acid ($HS(CH_2)_{10}CO_2H$) (11-MUA), ethanolamine ($H_2NCH_2CH_2OH$), hydrogen peroxide (H_2O_2.aq), ammonia (NH3.aq), phosphate buffered saline (PBS), acetate

buffer (0.1 M, pH 4.5), giemsa Stain, bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Steinheim, Germany) and were of analytical-reagent grade and used without any purification. Human CD133 PE-conjugated Antibody (FAB11331P), Human CD133 Antibody (MAB11331) were prepared from R & D system. In addition, the 50-nm gold layer deposited high-refractive-index glass (BK7) chips (25*10 mm) were purchased from Bionavis Company (Finland).

2.2. Methods

2.2.1. Isolation of mononuclear cells (MNCs) by gradient centrifugation

In the current experiment, we collected the samples from AML candidates represented to Shahid Ghazi Tabatabaei Hospital affiliated to Tabriz University of Medical Sciences. All patient were asked to complete informed consent. Bone marrow (BM) cells from AML patient were collected with the approval of the Research Ethics Committee of the University of Tabriz medical science with ethics code IR.TBZMED.REC.1396.1307. A total of 10 patient specimens were analyzed for a variety of leukocyte surface markers from February 2018 to August 2018. We enrolled the samples to our study which diagnosed with AML. In the current experiment, 7 patients with AML at the mean age of 50 years were studied. Isolation of human MNCs was done using Ficoll-Hypaque solution. First, 5 ml of diluted blood samples were gently overlaid to Ficoll-Hypaque solution and centrifuged at 400 for 20-40 min. Then, cells at interphase were collected gently using a sterile syringe and washed twice with PBS. Finally, MNCs were transferred to a sterile tube for subsequent analyses. Based on our preliminary analysis, we found 90% cell survival rate assessed by the Trypan blue exclusion test.

2.2.2. SPR instrument

For this propose, a multi-parameter SPR system (MP-SPR Navi 210A, BioNavis Ltd, Finland) was used for cell interaction analysis that uses the flow injection channels with various injection lines. The SPR test was probed in a fixed angle form after the first scan of gold surface mode with a flow rate of 20 µl min^{-1} at a fixed temperature 35°C. The wavelength of a laser source for exciting the surface plasmons on the gold dielectric surface was set to 670 nm. SPR-Navi 210A gold chips were prepared of BK7- glass slide with 250 mm^2 area containing sputtered 50 nm gold layers on it.

2.2.3. Washing the bare gold chips

Prior to the functionalization of the gold-sensor slide, gold chip surface contaminations were eliminated by soaking in ammonia/hydrogen peroxide solution. For this purpose, each gold chips was put in boiling solution of ammonia (NH_3.aq) and hydrogen peroxide (H_2O_2.aq) in Milli-Q-water for near 20 min at 100°C. Then, the gold chips were dipped in distilled water and ethanol solution followed by the pass from the nitrogen stream. This washing step removes any impurity on the gold surface.

2.2.4. The functionalization procedures of the *biosensor*

For the modification of SPR biosensors, the procedures were performed as described in our previous work [31]. Briefly (i) 2mM MUA solution was prepared in ethanol and distilled water solution with ratio of 7:3, respectively (ii) 300 µl of the diluted MUA ethanol solution was dropped on the surface of gold sensors and incubated at room temperature for 24 hours (iii) after washing with buffer and ethanol, the chips coated with EDC: NHS 1:1 (NHS 0.05 M+ EDC 0.2 M) solution for one hour to activate carboxylic groups; (iv) 25, 50 and 100 µg/ml CD133 antibody dissolved in acetate buffer (pH=5.5) was added on chips for 50 minutes (v) After washing with PBS, 0.5% BSA was used for 30 min to block non-immobilized surface on gold chips (vi) by rinsing and drying with nitrogen stream, the glass surface of each sensor slide was exactly cleaned with ethanol solution (vii) Finally, functionalized gold chips are ready for inserting in SPR slide holder to investigating interactions.

2.2.5. Real-time analysis by SPR

Prior to cell injection, freshly isolated MNCs from blood samples was counted using a cell counter device. A defined number of cells per ml was rinsed and re-suspended in running buffer. The whole flow path was washed and filled with the related running buffer in any section before the SPR experiments. All cell injections were carried out in PBS as the running buffer in pH 7.4 at 35°C at the flow rate of 20 µl per min. Following cell injection, the chips were rinsed with buffer to remove the unbound cells. Data were analyzed using data viewer and TraceDrawer™ 210A SPR Bionavis software. The flow chamber has dimensions of 2 mm × 5 mm×100 µm (width× length × height) and all cell injection was performed at a flow rate of 20 µl/ml. Before flowing the cell suspension, the system was primed with PBS as running buffer to eliminate air bubbles from the sample lines and flow channels. All SPR tests were done on the fresh CD133 antibody chips without the need to regeneration steps between the sample injections.

2.2.6. Flow-cytometry (FC) analysis

We also analyzed MNCs isolated from bone marrow by FC using phycoerythrin (PE)-conjugated antibody against CD133 marker. Other factors such as CD13, CD33, CD10, CD11b, CD45, CD34, CD38, CD117, and CD64 also analyzed to exactly address the tumor cell phenotype. For this propose, MNCs were blocked with 1% BSA for 20 min. Then, 1 µl of PE-conjugated anti-human CD133 antibody was added to cell solution and incubated for 1 h at 4°C. After twice washing, FC analysis was performed using a FACSCalibur (BD Bioscience) system and data were processed by FlowJo software ver.7.6.

2.2.7. May-Grünwald Giemsa (MGG) staining

The isolated cells were smeared on the glass slides and immersed in the May-Grünwald solution for 3 min. Then, the slides were transferred into a Coplin jar containing the buffer solution (pH=6.8) and maintained for 1 min. Thereafter, slides were incubated with Giemsa R solution (diluted to 1: 20 in pH 6.8 buffer solution) for 10 min. After washing with neutral water (pH=7), the slides were air-dried and visualized by Olympus microscopy.

3. Results and Discussion

3.1. Optimization CD133 antibody immobilization on SPR chips

As seen in **Fig 1a**, the surface of the bare gold chip was modified with MUA for the formation of a carboxylic layer. Following activation with EDC/NHS solution, the antibody was easily stabilized on the carboxylic groups using covalent amide binding formation. BSA has the potency to cover the unmodified molecules of the glass surface. Immobilization of antibody was performed in pH 5.5 of acetate buffer due to the positively charged proteins under isoelectric point (pI). Therefore, the negative charge of COOH groups on MUA surface may cause too the great electrostatic binding formation and increase the response on the modified gold surface. The level of obtained angle shifts in the SPR curve of the MUA-gold surface was compared to an antibody-immobilized sensor at the concentration of 25, 50, 100 µg/ml determined by 640, 360 and 190 millidegrees (**Fig 1b)**. SPR has able to measure any trivial mass and refractive index changes near of the gold sensor surface evident with shifts of SPR curves in an array format of the modified gold surface. Commensurate with these comments, the formation of MUA SAM and antibody immobilization was completed suitably on the gold sensor surface and the 25 µg/ml concentration of CD133 antibody had a more immobilization level on the gold surface. On the basis of the amide bonding formation between MUA and anti-CD133 on the SPR chip, they were ready to trap and detect target

cells by the SPR biosensor. **Fig 1c** outlined the bar graphs of SPR angle versus modified surface of the gold chip at immobilization steps.

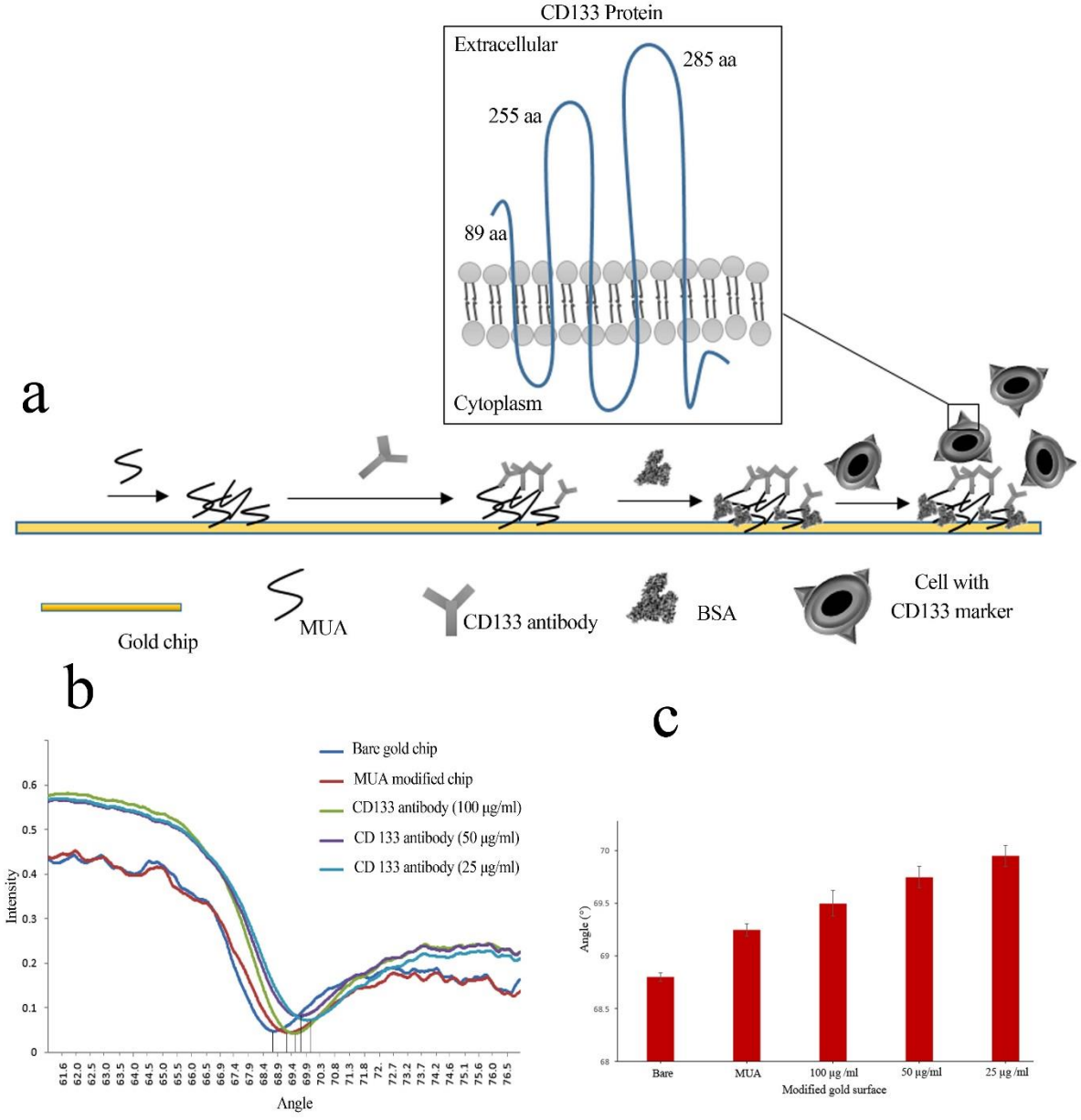

Fig 1: (**a**) A schematic illustration of immobilization of CD133 antibody on the gold surface (**b**) SPR curves shift in the modified gold surface after MUA modification and immobilization of anti-CD133 antibody (**c**) The bar graphs of SPR angle versus modified surface of the gold chip.

3.2. Characterization of CSCs by SPR biosensor

The capacity of attached ligands to capture specific cells and produce an SPR response is dependent on many factors. Some of the biological aspects like repulsion of charged agents on the negative MUA layer on the gold chip and negative cell surface determine the SPR signals after capturing cells onto the sensor surface [35]. Therefore, blocking the MUA layer with BSA molecules omits any charged groups on the sensor surface following the immobilization of CD133 antibody.

To determine the best capacity of immobilized ligands on the gold surface against cell binding, we examined the various concentration of cells injection on the target surface (1×10^5, 2×10^5 and 3×10^5 cells/ml). As shown in **Fig 2a**, the injection of 3×10^5 cells/ml on CD133-modified surface has been making fewer responses compared to 2×10^5 cells/ml counterpart. However, the introduction of 1×10^5 cells/ml had a detectable response for cell analysis. These results indicated that the capacity of the immobilized antibody on the sensor surface decreased when the cell number reached over the 2×10^5 cells/ml. We also found that the density of the immobilized antibody and cell surface marker can influence the quality of cell capturing. The flow rate during cell sample injection could orient cell affinity and/or detachment to the gold surface. So in this study, all samples injection was done at a stable flow rate of 20 µl/min. The selectivity and distinct affinity of the CD133 sensors to capture CSCs was also assessed using SPR. The biosensors were incubated with 1% BSA and response units were recorded. As shown in **Fig 2b**, no significant response was observed in BSA-treated chips exposed to various numbers of cells ranging from 1×10^5 to 3×10^5 cells per ml while an appropriate response was observed after introduction of samples on CD133-coated chips. So, for the minimizing of non-specific bindings, 1×10^5 cells/ml was introduced on CD133 modified gold chips during SPR tests. These results exhibit the suitable selectivity of the developed immunosensors for the detection of CSCs among various cell types. **Fig 2c** represents the cells affinity to chips coated with CD133 and BSA, displaying CSCs affinity to CD133 surface was 8-10 times more than that of BSA group. In line with these data, we confirmed that CSCs are specifically captured by an anti-CD133 functionalized surface.

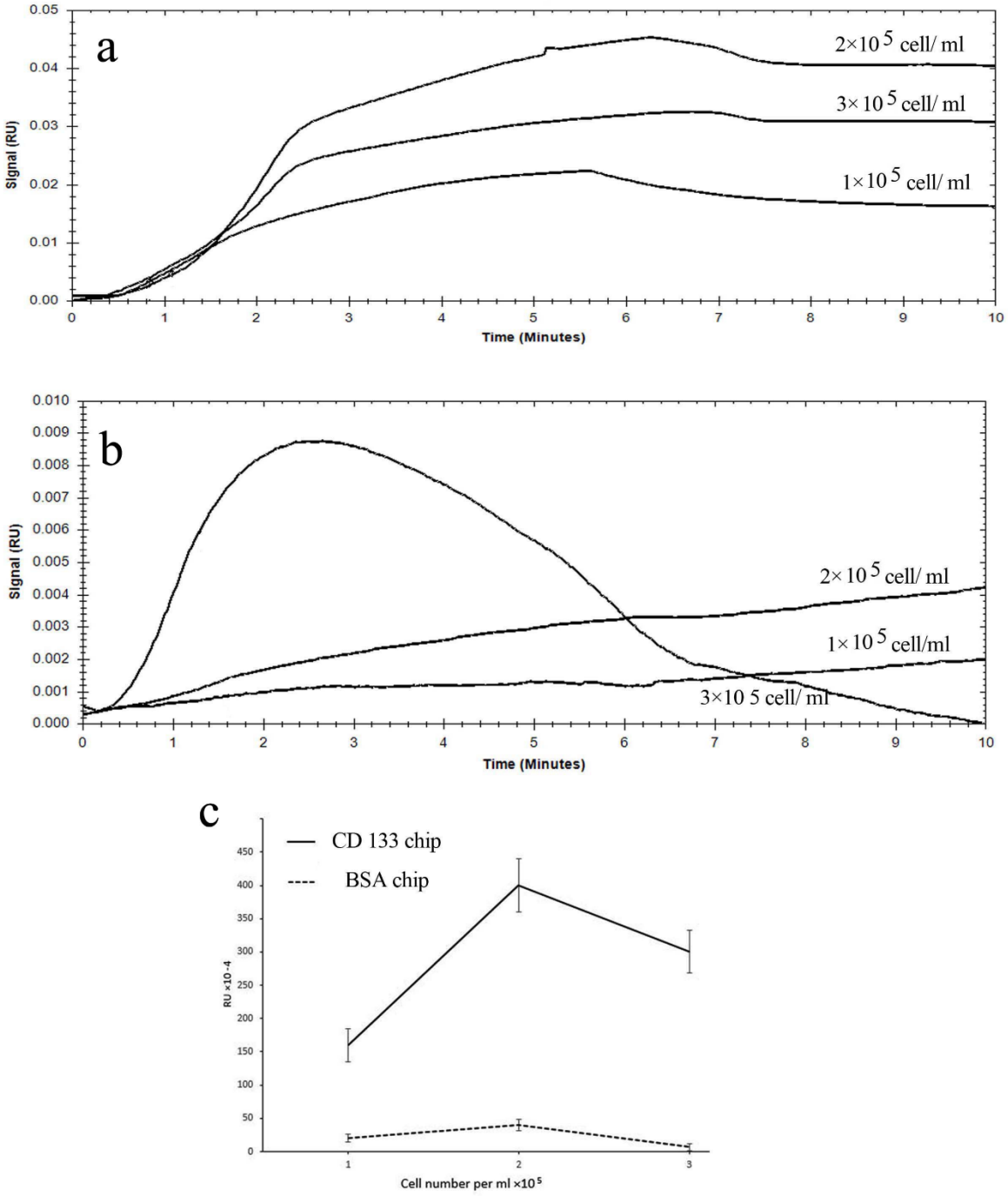

Fig 2. (**a**) SPR responses for different concentrations of cells on CD133 modified surface (**b**) SPR responses on BSA-modified surface for non-specific binding (**c**) The related curve between cell number and RU value in CD133 and BSA chips.

3.3. CD133 marked CSC detection: SPR & FC

The ability of SPR biosensors to detect CSCs was explored in the current experiment. We also examined the freshly isolated MNCs from BM (bone marrow) or PB (peripheral blood) of patients with AML based on the distribution of the CD133 marker. Sedimentation and capture of cells on the modified surface caused an increase in RI values. All positive cells captured and attached to CD133-antibody surface within 5 min. For this propose, 1×10^5 cells/ml was count and passed over a sensor with an immobilized CD133 antibody. The results revealed different CSCs density from AML patients based on diversity in RU and FC values. A sensorgram of the immunosensors (CD133) for the seven samples was shown in **Fig 3a**. RU was intensified by an increase of CD133 positive cells number captured on a gold surface, indicating a close relationship between the number of target cells and the intensity of SPR values **(Fig 3b)**. The biosensor was capable to detect the CSCs in cell population with cell density more than 1×10^5. The injection of same MNCs cells isolated from healthy person blood and injection PBS on CD133 chips show the RU value 60 ± 15 and 5 ± 2 respectively. These data indicate that the minimum of obtained RU value for detection of CD133 marked cells must be more than 75 RU (All RU values correspond to RU$\times10^{-4}$). More details about the cell spreading and sedimentation into the evanescent field during interaction cell surface marker and specific ligands on an SPR chip was presented by Stojanović and Schasfoort [25, 27].

To assess the relationship between SPR RU and CD133 marker density, SPR responses of MNCs were measured and compared to the specific fluorescence intensity determined with a FC analysis **(Fig 3c)**. Linear regression for the cell line data combined gave an R^2 of 0.96 (**Fig 3d**), indicating that feasibility of SPR for immunophenotyping of distinct cells. Additionally, SPR signals are dependent on CSCs concentration, size and mean CD133 antigen density. Quantitative CD133 analysis in CSCs is beyond reach since the correlation of the particle amounts and dimensions were not determined by the SPR response. SPR signals related to mean CD133 antigen density on the cell surface. Moreover, SPR detected lower antigen levels better than FC because SPR measures one side of the CSCs group binding to the modified sensor surface, whereas FC detects all side of flowed single CSCs.

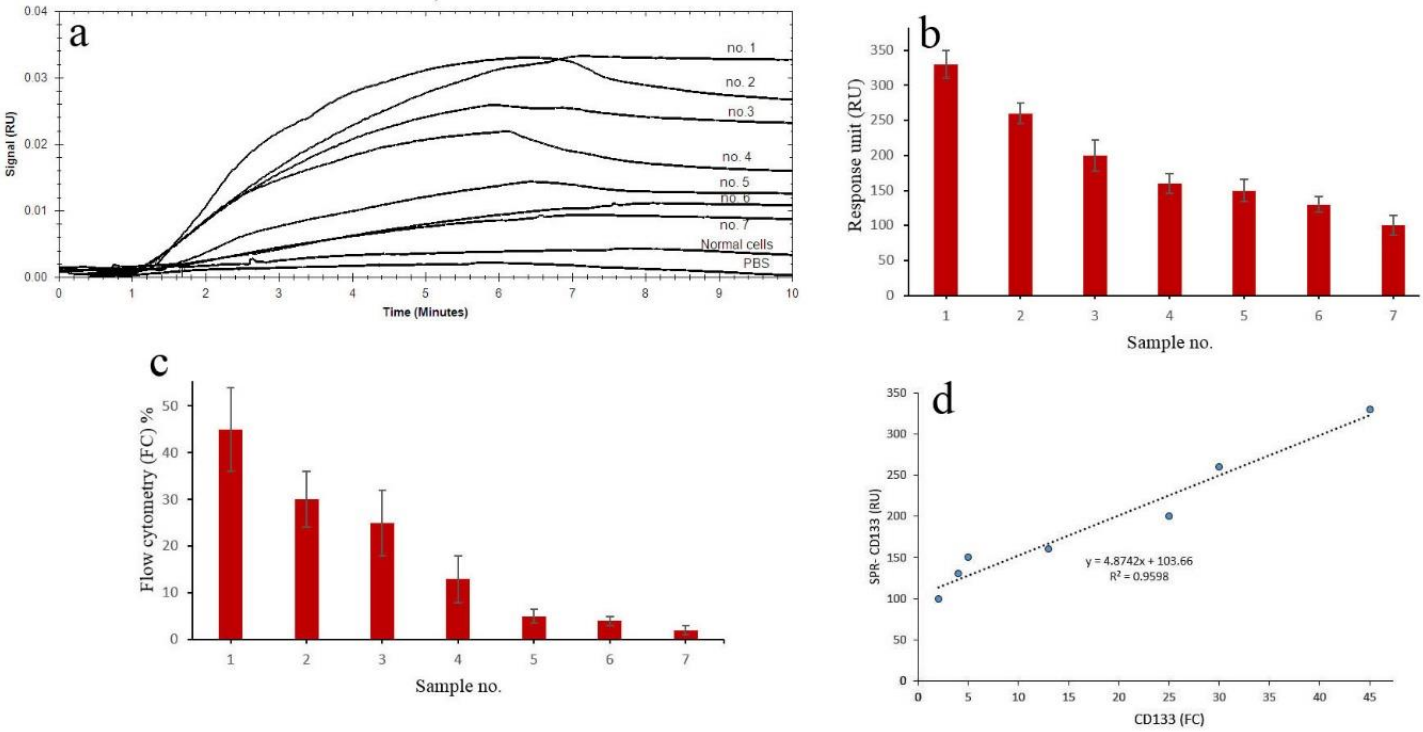

Fig 3: (**a**) Sensorgrams obtained from the injection of 1×10^5 cells onto pre-incubated chips with anti-CD133 in various AML patients (at flow rate of 20 µl per min; injection time 5 min) (**b**) The bar graphs of SPR responses versus different samples (**c**) The bar graphs of FC analysis versus different samples (**d**) Correlation between antigen detection by SPR and FC, performed for MNCs of different AML. SPR signals correlated with the specific fluorescence intensity (SFI) of FC with linear regression for antibody– cell combinations. The corresponding r^2 were determined on the overall correlation ($r^2 = 0.96$). Error bars show the standard deviations for three replicates.

Based on the panel of CD marker done by FC, we found an increased number of progenitor cells which were positive for CD117, CD45 and CD34 and CD33, confirming the occurrence of myelomonocytic proliferation (**Table 1**). In line with these changes, the level of CD133 was found to increase both in BM and PB. However, we found a large number of CD133 positive cells in samples prepared from BM. Although, the percent of CD133 positive cells was less in the systemic circulation, SPR efficiently had potential to detect easily the presence of cells with multipotential. The low number of CD133 positive cells in blood even after the occurrence of various BM tumors causes difficulty in interpreting the data obtained by FC. In addition, CD117, but not CD133, is commonly used to measure the number of progenitor cells in subjects with BM tumors. The CD117 molecule is a transmembrane protein receptor more frequently found on stem cell, leukemic hematopoietic cells and in AML [36]. This marker is touted as a basic myeloid marker in all subtypes of AML (M0 to M5). An increase in the number of $CD34^{+}$ subpopulation, a primitive stem cell marker, shows a poor outcome. Specific expression of different CD45 can be seen in numerous stages of differentiation of normal nucleated hematopoietic cells [36]. The emergence of CD45 cells in AML confirmed the expansion of myelocytic lineages and specifically are modulated in each AML subtype. In the current experiment, we aimed to measure the existence of progenitor cells, CD133 cells, after the onset of AML by two approaches, FC analysis and SPR test. Following tumor development, progenitor cells directly or indirectly are affected. So, designing a novel approach to precisely detect the presence of CD133 enables us to diagnose, interpret and monitor the tumors expansion and recession. At the recent research in 2017, antigen profiling of extracellular vesicles using the SPR method was analyzed and the results revealed the correlation between SPR and FC signals in MCF7 cells ($R^2 = 0.78$) [37].But the corresponding r^2 in this work were determined on the overall correlation with $R^2 = 0.96$ for CD133-marked cells.

"Table 1"

3.4. May-Grünwald Giemsa (MGG) staining

The microscopic examination revealed the existence of myeloblastic progenitor cells in AML subjects (**Fig 4)**. In normal condition (**Fig 4h)**, progenitor cells are not detectable in PB. In response to an increased body demand and/or the occurrence of different BM tumors, these cells proliferate without control and occupy the effective hematopoietic microenvironment and eventually enter the systemic blood system. In most cases, the progenitor cells have a great proliferation potential and distinct cell morphology. The presence of typical dark blue or purple staining cytoplasmic region indicate the rate of nucleus/cytoplasm is increased as shown in our slides (arrows in **Fig 4**). In the nucleus, different nucleoli are observable coincided with the emergence of cytoplasmic granules. The number of myeloblasts, promyeloblasts, metamyeloblasts, and undifferentiated myeloblasts were shown to increase in AML candidates. Light microscopic examination of a bown marrow or peripheral blood stained with Giemsa is a rapid, but insensitive method for diagnosis hematological malignancies compared with FC and SPR method. Although the Giemsa stained method provides qualitative information however requires trained personnel but who can differentiate unusual cells from artifacts detected on samples [38, 39].

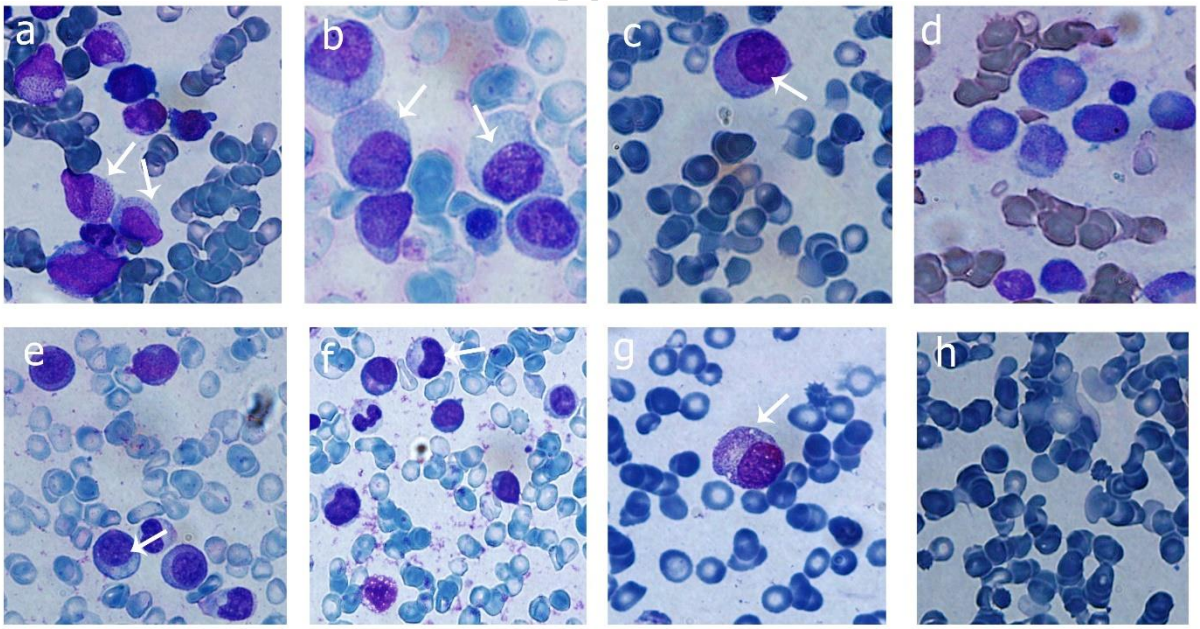

Fig 4. Microscopic slides stained with May-Grünwald Giemsa solution. (a, b, c, d, e, f, g & h is related to sample no 1, 2,3, 4, 5, 6 ,7 & normal sample)

5. Conclusion

Here, we developed a label-free SPR biosensors for the detection of CSCs using CD133 cell surface markers. Further precise development of these immunosensor types will simplify and

strengthen the detection of CSCs within cancer cells which thought to be important in tumor resistance and help clinicians in monitoring AML progress and cancer therapy. The dependence of the SPR RU to cell concentration was determined with serial dilutions of freshly isolated MNCs from AML candidates on the gold sensor surface. Based on the results, the gold slides of the SPR sensing chip loaded with 25 µg/ml CD133 antibody had the most angle shift and the 1×10^5 cells/ml had the best capture capacity at the flow rate of 20 µl/min. Both SPR and FC responses correlate with mean antigen density (R^2= 0.96) and SPR had appropriate sensitivity in monitoring and detecting distinct cell type in AML subjects. We exhibited that immunosensors is suitable for the analytically sensitive and qualitative detection of CSCs by the capturing target cells on the specific surface. Calling attention, the SPR biosensors as diagnostic approaches are able to overcome the complexity of such FC analysis, allowing to monitor a real-time signal for specifying the cell surface markers without the need of multiple-label staining steps in the context of cancer biology.

Conflicts of interest

There are no conflicts to declare.

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Tables:

Table. 1. Monoclonal antibodies and CD133 expression in AML cells (All RU value correspond to RU×10^{-4})

Sample no	CD13	CD33	CD10	CD11b	CD45	CD34	CD38	CD117	CD64	CD133*	SPR value (RU) *
(BM) 1	46%	64%	neg	31%	82%	neg	84%	30%	25%	45%±9	330± 20
(BM) 2	6%	98%	neg	neg	99%	neg	100 %	92%	92%	30%±6	260± 15
(PB) 3	neg	79%	neg	13%	98%	neg	77%	66%	neg	25%±7	200± 22
(BM) 4	55%	neg	neg	23%	84%	5%	55%	51%	neg	5%±5	150± 16
(BM) 5	76%	neg	neg	neg	91%	neg	94%	84%	neg	13%±1 .5	160± 14
(PB) 6	25%	45%	neg	65%	98%	4%	71%	4%	33%	4%±1	130± 11
(BM) 7	23%	neg	neg	23%	98%	15%	49%	29%	neg	2%±1	100± 14
(PB) Normal	16%	8%	4%	13%	52%	neg	35%	5%	10%	3%±1	60±1 5

* Investigated value using our method
AML: acute myeloid leukemia, BM: bone marrow, PB: peripheral blood, RU: response unit

Highlight

- SPR-based method was developed for detection of $CD133^+$ cancer stem cells in patients with AML.
- SPR was validated by using CD133 antibody concentrations and cell density.
- A strong correlation was obtained between SPR and flow cytometry responses.

Graphical abstract