Biophysical Differences between Chronic Myelogenous Leukemic Quiescent and Proliferating Stem/Progenitor Cells

Nataliia V. Guz¹, Sapan J. Patel^{1,2}, Maxim E. Dokukin³, Bayard Clarkson², Igor Sokolov^{3,4,5*}

¹ Department of Chemistry, Clarkson University, 8 Clarkson Avenue, Potsdam, New York 13699-5820, USA

² Memorial Sloan Kettering Cancer Center, Sloan Kettering Institute, Molecular Pharmacology and Chemistry Program, 415 East 68th Street, New York, NY, 10065

³ Department of Mechanical Engineering, ⁴ Department of Biomedical Engineering, ⁵ Department of Physics and Astronomy, Tufts University, 200 Boston Ave, Medford, MA, USA;

Short title: Biophysical differences of leukemic quiescent and proliferating stem/progenitor cells.

Corresponding authors: Igor Sokolov, Department of Mechanical Engineering, Tufts University, 200 Boston Ave, Medford, MA, USA; phone 1-617-627-2548; email: igor.sokolov@tufts.edu; Bayard Clarkson, Memorial Sloan Kettering Cancer Center, 415 East 68th Street, New York, NY, 10065, USA; phone 1-646-888-2080; email: clarksob@mskcc.org

This work was supported in part by The Enid A Haupt Charitable Trust, the MeadWestvaco Corp and The E./S. Sindina Lymphoma Research Fund to B.D.C., the NIH/NCI Cancer Center Support Grant P30 CA008748 at MSKCC (B.D.C.) and NSF grant CMMI 1435655 (I.S.).

The authors have no conflicts of interest to declare.

Word count for the abstract: 150

complete manuscript word count (body text and figure legends): 5053

number of references: 60

number of figures/tables: 3/2

Abstract

The treatment of chronic myeloid leukemia (CML), a clonal myeloproliferative disorder has improved recently, but most patients have not yet been cured. Some patients develop resistance to the available tyrosine kinase treatments. Persistence of residual *quiescent CML stem cells* (LSC) that later resume proliferation is another common cause of recurrence or relapse of CML. Eradication of quiescent LSCs is a promising approach to prevent recurrence of CML. Here we report on new biophysical differences between quiescent and proliferating CD34+ LSCs, and speculate how this information could be of use to eradicate quiescent LSCs. Using AFM measurements on cells collected from four untreated CML patients, substantial differences are observed between quiescent and proliferating cells in the elastic modulus, pericellular brush length and its grafting density at the single cell level. The higher pericellular brush densities of quiescent LSCs are common for all samples. The significance of these observations is discussed.

Keywords: Chronic myeloid leukemia; atomic force microscopy; cell mechanics; personalized medicine; single cell analysis

Background

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder^{1 2} accounting for 1–2 cases per 100,000 in adults . Recent development of tyrosine kinase inhibitors (TKIs) have changed the prognosis of chronic phase CML from a life threatening disease into a treatable chronic disease with substantially increased survival.^{3 4} However, some patients develop resistant mutant clones^{5 6}, and there are a substantial number of cases of recurrence of CML upon TKI withdrawal^{7, 8, 9}. Various mechanisms have been proposed to better understand leukemia stem cell (LSC) populations that are responsible for recurrence or relapse. Persistence of residual leukemic quiescent stem cells and development of resistant clones are the most probable causes.^{2, 10, 11} It is still not clear how to eradicate surviving quiescent LSC which are resistant to many types of treatment².

Because LSCs are quite rare, it is hard to apply standard biochemical methods such as western blotting to study these cells. Thus, it is important to develop new methods that allow reliable measuring of the cell properties at the single cell level. Atomic force microscopy (AFM) is a biophysical technique capable of measuring physical properties of single cells^{12, 13}. Typically, AFM can function as a microscope for imaging viable cells^{14, 15} and even single molecules¹⁶. The AFM technique is also one of the most convenient methods for studying the mechanics of soft materials in general¹⁷, and cell mechanics in particular^{18, 19, 20, 21} because it can operate with a large range of load forces at small scales with very high accuracy²².

Over the last decade, many studies have demonstrated the link between cell mechanics and various diseases, such as cancer^{20, 21, 23, 24}, arthritis²⁵, malaria²⁶, ischemia²⁷, and even aging^{19, 28, 29}. The careful analysis of cell mechanics may enable researchers to obtain new fundamental

1
2
3
4 insights of disease states as well as properties of different cells within normal or diseased cell
5
6 populations, and help to develop improved methods of diagnosis, prognosis, and treatment.
7
8

9 It is important to develop methods that allow accurate measuring of mechanical
10
11 properties of cells independently of the specifics of the used methods and instruments. It has
12
13 been shown that the elastic modulus (*aka* the effective Young's modulus) and the parameters of
14
15 the pericellular coat (*aka* the pericellular brush layer) can be such objective characteristics of
16
17 cells ³⁰. These characteristics can be experimentally measured with the help of AFM.
18
19
20

21 To extract the instrument and material independent biophysical characteristics of cells,
22
23 the force indentation data has to be analyzed with the help of a mechanical model. The Hertz
24
25 model ³¹ and its various modifications ^{23, 32} have been widely used to determine the elastic
26
27 modulus of cells. In these models, the cell is considered as a homogenous and isotropic material,
28
29 and the cell shape is not often taken into account. However, the cell surface is typically non-flat
30
31 even at the nanoscale. Various membrane protrusions, which can be seen in optical confocal
32
33 microscopy (see, e.g., ²⁰) can be detected with AFM ³³. It has recently been found that the
34
35 pericellular brush interferes with indentation measurements of elastic properties of the cell body,
36
37 and a new model must be used ³⁴ which separates contribution of the pericellular brush layer and
38
39 deformation of the cell body in the AFM indentation experiments. Interestingly, cancer cells may
40
41 look artificially softer if the cellular brush is not taken into account as was shown for the case of
42
43 human cervical epithelial cells ²⁰.
44
45
46
47
48
49

50 Furthermore, as was recently shown ³⁰, cells can be treated in a self-consistent way as an
51
52 elastic isotropic and homogeneous material (cell body) surrounded by essentially non-elastic
53
54 pericellular brush layer which cannot be described by the elastic modulus. The non-elastic brush
55
56 layer demonstrates the exponential force behavior somewhat similar to the classical polymer
57
58
59
60
61
62
63
64
65

entropic brush. Therefore, a term “pericellular brush” was introduced to describe this layer. One can characterize the mechanical properties of cells with three parameters, the elastic modulus, the (equilibrium) length and grafting density (effective number of molecular constituents per unit area) of the pericellular coat/brush.

The above approach to characterize cell mechanics is used in the present work to study critical differences between CML quiescent (G_0) and proliferating (G_1) stem and progenitor (S/P) cells. Hereafter G_1 is used to include cells in all phases of the cell cycle except G_0 , including G_1 , S, G_2 and M phases. In CML the primary driving mutation is a fusion of the Abelson murine leukemia (ABL) gene on chromosome 9 with the breakpoint cluster region (BCR) gene on chromosome 22 (Philadelphia chromosome; Ph⁺)³⁵, which results in expression of a fused oncoprotein, termed BCR-ABL. The BCR-ABL oncoprotein is a constitutively active tyrosine kinase that promotes growth and replication through downstream pathways². To study differences in quiescent and proliferating CML stem/progenitor cells, we enriched patient samples for CD34⁺ cells using immunophenotypic selection from CML blood samples³⁶. Although the CD34⁺ cell fractions are heterogeneous, they contain the great majority of S/P cells and have been shown to reconstitute the entire hematopoietic system of immunocompromised irradiated mice^{37, 38, 39}.

Both populations of proliferating and quiescent cells were subjected to AFM analysis to measure the biomechanical properties described above at the single cell level. We observed statistically significant differences between quiescent and proliferating cell populations for both the Young’s modulus (cell “stiffness”) of the cell body and the parameters of the pericellular brush. Only one basic parameter, the brush grafting density, showed a common behavior for all four patients, the grafting density. It was higher for the quiescent cells compared to the

proliferating ones (though this difference was not statistically significant for one of the patients). The same behavior was observed for two derivative parameters, the brush “size” and “volume density”. The significance of these results for prognostics and future treatment of this type of cancer is discussed.

Methods

CML patient samples

CML blood samples were obtained from four patients newly diagnosed with chronic phase CML before any treatment. The patient samples were obtained from Human Blood Bank Facility (HBUC) at MSKCC for research purpose with approval number HBUC# HBS2012091. Some of the patient samples were gifts from Dr. David Scheinberg which were obtained with informed consent for research purpose. Some patient samples were from Dr. Clarkson’s lab which were obtained between 1986-1990 with informed patient consent for research purpose. The CML blood samples were processed to obtain enriched peripheral blood mononuclear cells (PBMCs) and frozen until further use. After informed consent on Memorial Sloan-Kettering Cancer Center (MSKCC) Institutional Review Board–approved protocols, PBMCs from patients were obtained by Ficoll density centrifugation. Defrosted PBMCs from the four CML patient samples were individually used to isolate the CD34+ fraction using the midiMACS immune-magnetic separation Kit from Miltenyi (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat# 130-0460701). The details of CD34+ cell enrichment and Hoechst 33342 and pyronin-Y staining for G₀ cells enrichment⁴⁰ are described in the Supplementary information. The cells described above were plated on culture dishes. The AFM study was done on these live cells (the apical part, similar to^{41,42}) directly in the growth medium without any modifications, see the Supplementary

information for detail.”

Deformation of stem cells with the AFM probe: A model for a spherical cell covered with pericellular brush layer

It has been demonstrated that the use of a two-layer model (cell body plus pericellular “brush” layer) is more accurate than the classical Hertz model (and the only self-consistent approach when measuring mechanical properties of cells³⁰. In the other words, the brush model allows one to extract the elastic, Young’s modulus of the cell body in a quantitative manner. When the AFM probe deforms a cell, both pericellular layer and cell body are squeezed. The brush model considers it. The model is applied to the analysis of force indentation curves recorded with AFM in two steps. First, the part of the force curve near the maximum load force is analyzed. This part of the curve should correspond to an almost fully compressed pericellular brush layer. Otherwise, the maximum load force should be increased. Thus, it corresponds to the deformation of the cell body, which is considered to be an elastic homogeneous and isotropic material (this assumption is verified, for example, by testing independence of the modulus of the indentation depth, see, Fig.S6) . The Young’s modulus (or just elastic modulus) of the cell body is derived from that part of the curve. The knowledge of the elastic modulus allows one to extrapolate the cell body deformation to smaller deformation forces. In the second step, the force due to the pericellular brush is derived and analyzed. Our previous studies have shown that this force is well fitted with the exponential function, and can be well understood in terms of polymeric brush model^{30, 43, 44}. In principle, one could consider the exponential law brush behavior as just a parameterization of the force dependence of this outside layer. Considering different

parameterizations would not qualitatively change the conclusion about the observed difference of that top (pericellular) layer.

The brush model was further extended to the case of cells loosely attached to the bottom of the culture dish⁴⁴. LSCs are indeed only loosely attached to the bottom of the culture dish. One can clearly see this under regular optical microscope by gentle knocking the culture dish and observing the cells moving. Since this model was described previously⁴⁴, it is just briefly outlined below. Figure 1 shows a scheme of the interaction of a spherical AFM probe with a cell represented as a double two-layer structure. Geometry presented in Figure 1 implies:

$$Z_0 - Z - d = (i_{top} + i_{bottom}) + (h_{top} + h_{bottom}), \quad (1)$$

where Z is the relative vertical scanner position. The position $Z=0$ was chosen for Z position corresponded to the maximum cantilever deflection d . Z_0 is the scanner position for the non-deformed state of the cell body, h_{top} is the separation distance between the cell body and the spherical probe, h_{bottom} is the distance between cell body and the substrate, i_{top} and i_{bottom} are the top and bottom deformation of the cell body, correspondingly. Eq.(1) can be read the total “indentation” depth measured by AFM ($Z_0 - Z - d$) equals to the cell indentation magnitudes $i_{top} + i_{bottom}$ minus the size of the brush layer ($h_{top} + h_{bottom}$).

It was assumed that near maximum indentation force (maximum deflection) the cell’s brush is completely squeezed and $h = h_{bottom} + h_{top} = 0$ (see the Supplementary information on justification of this approximation). This allows using Hertz model to calculate Young’s modulus E of the cell body from the observed deformation i :

$$i = Z_0 - d - Z = d^{2/3} \cdot \left[\frac{3}{4} \frac{k(1-\nu^2)}{E} \sqrt{\frac{R_{probe} + R_{cell}}{R_{probe} R_{cell}}} \right]^{2/3}, \quad (2)$$

where $i = i_{top} + i_{bottom}$, k AFM cantilever spring constant and R_{probe} and R_{cell} are the radii of the AFM probe and cell body, correspondingly. The Poisson ratio ν of a cell was chosen to be 0.5 for all calculations in this work. A specific choice of this parameter does not change the conclusions of this work, neither changes the Young's modulus beyond the variability of the obtained results. Radius of each cell was determined from the topography images collected within the force volume mode which were corrected for the cell deformation (the height of each pixel was increased by the value of i).

With known cell deformation, the force-separation dependence $F(h)$ can be reconstructed using equation (1):

$$F(h) = kd = k(h - Z + Z_0 - i) \quad (3)$$

To describe the brush parameters quantitatively, the following steric interaction equation can be used⁴⁴:

$$F(h^*) \approx 50(k_B T) R^* L N^{3/2} \exp\left(-2\pi \frac{h^*}{L}\right), \quad (4)$$

where k_B is the Boltzmann constant, T is temperature, $h^* = h_{top} + h_{bottom}$,

$R^* = 1/2 \cdot R_{cell} \sqrt{R_{probe}/(R_{probe} + R_{cell})}$ is the effective radius N is the surface density of the brush constituents, i.e., the effective number of molecular constituents per unit area (grafting density), and L is the equilibrium thickness of the pericellular brush layer surrounding the cell body. This formula is valid for $0.1 < h/L < 0.8$. Following⁴⁴, we assumed that N is a geometrical average of the top and bottom densities ($N = \sqrt{N_{top} N_{bottom}}$) and $L/2$ is equal to the top and bottom brush length ($L = L_{top} + L_{bottom}$). Here we do not differentiate between bottom and top brush layers.

Results

The approach part of the force curve was used for calculation (constant vertical speed of $\sim 10 \mu\text{m}/\text{sec}$). This speed was a compromise between desire to minimize viscoelastic contributions and disturbance of the cells by the AFM probe. It is conceivable that the obtained parameters still contain viscoelastic contribution. Thus, it is important to keep this speed the same in all measurements. It has to be noted that the speed used in this work is quite ordinary when studying cell mechanics⁴⁵. Furthermore, as was demonstrated for neurons, the mechanics of the cell body is virtually speed independent within $1\text{-}10 \mu\text{m}/\text{sec}$ ⁴⁶ (whereas the parameters of the brush do depend on the ramp speed).

An example of the processing of the AFM data for G_0 cells of patient 4 is shown in Figure 2. Figure 2a shows the first part of the brush model, the Hertz fitting (eq.(2)) which gives the Young's modulus of the cell body (2.3 kPa this particular example). One can clearly see that this fitting is good for higher forces but it deviates from the experimental data for small forces (small deflection of the AFM cantilever d). This is expected because the pericellular brush is squeezed only at higher forces ($2\text{-}4 \text{ nN}$ in the case of leukemic cells). This is in agreement with the behavior observed in previous work which we used the brush model³⁰. The deviation between the Hertz fitting and the experimental data (Figure 2a) for small forces is attributed to the steric force due to the pericellular coat.

The repulsion force between the AFM probe and pericellular brush layer (eq.(3)) is presented in Figure 2b. Exponential behavior can be clearly seen as a straight line in the logarithmic scale in this figure. This data is further processed with equation (4) to calculate the grafting density N and the length of the pericellular brush layer L are found using eq.(4) ($L=456 \text{ nm}$, and $N=335 \mu\text{m}^{-2}$ in the specific example).

Figure 2 (c and d) demonstrates a typical inconsistency when the presence of pericellular brush is ignored. Figure 2c shows a substantial deviation from the Hertz model from the experimental data when fitted for small forces. Figure 2d shows also a poor Hertz fitting when trying to fit the entire force range. The fittings were calculated by using the standard nonlinear least square algorithms.

Mechanical properties of CD34+ G_0 and CD34+ G_1 CML patient cells:

We investigated biophysical properties of CD34+ G_0 and G_1 CML cells obtained from four untreated patients. Details of enrichment of cells collected from each patient with flow cytometry are presented in supplementary Table S1. The amount of cells and force curves processed here are summarized in Table 1. All these data were analyzed as described in the previous section.

The detailed results in the form of histograms are shown in the Supplementary Materials (Figs.S1-S4). Figure 3a shows the results of average (as well as the standard error of mean (SEM) values) of the Young's moduli for each patient sample. Similar averaged data for the length and grafting density of the pericellular brush are presented in Figures 3b and c, respectively. Figure 3d shows the effective "size" of the brush ($N*L$ parameter). The meaning of the latter parameter can be described as an effective total length of the brush molecules per unit area. This parameter would be equal exactly to the total length of those molecules if the brush was comprised of fully stretched molecules. In reality, the brush molecules are bent and coiled by the Brownian motion, and the total length is determined by many parameters, including the type of physiological solution.⁴⁷ Following the same logic, one can introduce a parameter of the effective volume "molecular density" of the pericellular brush layer, N/L . this is shown on Fig3e.

The quantitative results for the values of the Young's modulus and the brush parameters for all samples shown in figure 3 are presented in Table 2. There is an increase in elastic modulus for CD34+ G₀ (quiescent) compared to CD34+ G1 (proliferating) cells for patients 1. However, the elastic modulus of CD34+ G₀ cells is substantially lower than that of CD34+ G1 cells for patients 3 and 4. We did not observe any significant difference in the elastic modulus between CD34+ G₀ and G1 cells for patient 2. Further, we observed a substantial increase of the brush length (L) for CML CD34+ G1 cells compared to G₀ cells collected from patients 1, 2, and 4. However, the brush length of G₀ cells was higher than G1 cells for patient 3. Interestingly, one can see that the grafting density behaves similarly for all patients. CD34+ G₀ cells demonstrate higher grafting density than CD34+ G1 cells, though it is statistically significant for patients 1,2,3 only. Another parameter characterizing the effective amount of the pericellular brush layer (brush size, $N*L$) per unit area also showed that it is higher for CD34+ G₀ than CD34+ G1 cells for all patients. Similarly to the grafting density behavior, this difference is not statistically significant for patient 4. The average effective volume “molecular density” of the pericellular brush layer, N/L , is higher for CD34+ G₀ than CD34+ G1 cells for all patients. Note that distributions of this parameter are strongly non-Gaussian for patients 3 and 4, Fig.S4.

Discussion

Chronic myeloid leukemia (CML) has not been eradicated as yet. There are a substantial number of cases of recurrence or relapse of CML after cessation of treatment with imatinib or other TKIs. One of the most plausible mechanisms which leads to CML recurrence/relapse is the survival of a small number of residual leukemic *quiescent leukemic stem cells* (LSC) that later

1
2
3
4 resume proliferation. Eradication of such cells is a promising approach to prevent recurrence of
5
6 CML. The study of biophysical differences between quiescent and proliferating LSC may help in
7
8 providing new knowledge to attack the problem of eradicating quiescent LSCs.
9

10
11
12 Due to a rather limited number of such cells, it is important to develop experimental
13
14 methods which deal with studying single cells. Here we present utilization of such a method by
15
16 using AFM to study the biophysical properties of cells. Specifically, the Young's modulus
17
18 (characteristic of cell "stiffness"), pericellular coat/brush length, and its grafting density were
19
20 studied at the level of the single cell for LSCs obtained from four untreated chronic CML
21
22 patients. We observe that quiescent and proliferating LSC are significantly different from a
23
24 biophysical point of view. At the same time, we found a substantial variability between different
25
26 cancer patient samples. This is quite expected due to known heterogeneity of cancer cells.
27
28 Variation between patients may be attributed to either clinical heterogeneity ⁴⁸, molecular
29
30 heterogeneity ⁴⁹, or additional translocations that can affect BCR/ABL expression levels which
31
32 was observed by other researchers ⁵⁰. At the same time, the reasons for the biophysical
33
34 differences between quiescent and proliferating cells remain unclear. The results presented in
35
36 this work demonstrate previously unknown biophysical differences. Quiescent cells show a
37
38 significantly denser pericellular brush layer. This was statistically significant for three patients
39
40 when using grafting density and the brush size parameters. The cells collected from the fourth
41
42 patient demonstrated the same trend but the difference was not statistically significant. The
43
44 average values of the brush volume density parameter were noticeably different for all four
45
46 patients, Table 2. However, the distribution of this parameter was strongly non-Gaussian for
47
48 patients 3 and 4. The nonparametric Mann Whitney test showed no statistically significant
49
50 difference between these parameters of G0 and G1 cells.
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

To understand the significance of the presented biophysical results and their possible correlation with biochemical differences, let us first discuss the difference observed in the Young's modulus of the cell body. Quiescent cells of patient 1 were significantly stiffer compared to the proliferating cells. However, the opposite behavior was observed for patients 3 and 4 and no significant difference was observed for patient 2. From a biochemical point of view, there is considerable evidence that the modulus is defined by the cellular cytoskeleton^{12, 19, 51}, mostly by F-actin and its organization at the mesh layer of cytoskeleton [47-49]. Therefore, the presented results may be indicative of the differences in F-actin amount and its organization in quiescent and proliferating cells. This knowledge may be supplementary to the study of the role of the cytoskeletal proteins in cancer development. A number of alterations in the cytoskeleton of CML cells have been reported, some of which may affect different functions of these cells such as cellular movement, binding of chemo-attractants, and the cell cycle. One of possible mechanisms connecting these changes could be as follows. The BCR–ABL fusion protein is a tyrosine kinase⁵². BCR–ABL binds to actin and phosphorylates cytoskeletal proteins⁵³, and consequently, could correlate with the change of the Young's modulus. Another possible mechanism could relate to LMW-PTP (low molecular weight protein tyrosine phosphatases) whose presence has been correlated with poor prognosis in some cancers⁵⁴. LMW-PTP is a family of proteins associated with cytoskeleton rearrangement, and any differences in their levels could presumably also cause differences in the Young's modulus. Thus, measurements of the Young's modulus might be linked to the amount of BCR–ABL and/or LMW-PTP enzymes, and consequently, be considered as a relatively simple and fast method to measure it at the single cell level.

1
2
3
4 It should be noted that simple fluorescent staining of F-actin is insufficient to obtain
5
6 accurate information about organization of the mesh layer of F-actin as this requires the use of a
7
8 special AFM-based imaging method to derive it ¹⁹. In considering using those methods instead of
9
10 the approach described in this work, the present method of measuring cell mechanics seems to be
11
12 simpler and faster. It is also interesting to note that changes in stiffness of leukemic cells have
13
14 been recently observed ⁵⁵ for cells treated with chemotherapeutic drugs. Although the
15
16 measurements in that work were carried out using a simplified model and a sharp AFM probe,
17
18 the difference in cell stiffness/elastic modulus was substantial enough that the result would
19
20 presumably remain qualitatively the same compared to the more accurate brush model approach
21
22 used in the present work. However, it should be noted that it might be highly subjective to
23
24 compare parameters derived in an approximate model. In addition, the model used in ⁵⁵ does not
25
26 allow one to extract parameters of the pericellular brush layer.
27
28
29
30
31
32

33
34 To understand the significance of the observed differences in the pericellular coat, it is
35
36 instructive to describe what specifically is measured with AFM. AFM only measures the
37
38 physical presence of brush-behaving structures near the cell surface and does not discriminate
39
40 between the glycoproteins and polysaccharides in the glycocalyx or the membrane protrusions
41
42 and corrugations such as microvilli, microridges or filopodia. The significance and biological
43
44 functions of the pericellular brush are far from being fully understood ^{56, 57}. The glycocalyx is
45
46 known to surround neurofilaments to maintain interfilament spacing ⁵⁸, and the pericellular coat
47
48 on living cells is known to be responsible for cell-cell communication and interaction, cell
49
50 migration, differentiation, and proliferation ^{59, 60}. The size of the pericellular coating
51
52 (proportional to our parameter L) has been shown ⁵⁷ to correlate with the degree of invasiveness
53
54 of cancer cells although it's still not clear whether the brush size or its molecular composition, or
55
56
57
58
59
60
61
62
63
64
65

perhaps both, play the major role.

The size and density of the pericellular brush could also be used for prognostics of the stage of cancer progression of malignant cells. Based on biophysical observation, a larger brush layer would make it harder for a cell to penetrate through endothelial layers and enter tissues, i.e., it should decrease the cell's invasiveness. Significance can also be in the area of the development of cancer drugs. A substantial pericellular brush can work as a "filter" that captures drugs, and thus, prevents drugs from entering the cell. At the same time, if the brush has a large microvillus/microridge component it could result in higher efficacy of cancer drugs because of heigher surface area of such a cell, and consequently, its ability to interact with the drugs. Furthermore, we can speculate that the potential treatment based on the eradication of quiescent CML stem cells should definitely take into account the observed high density of the pericellular brush layer surrounding the cells. If molecules of potential drug are repelled by the pericellular layer, the efficacy of such drug would be a rather low, and vice versa.

To conclude, this study shows the use of the AFM method as a novel tool for evaluating and sensing changes in cancer cells, in particular, leukemia stem cells, when cells change their phenotype between quiescent and proliferating ones. The observed substantial difference in the Young's modulus and the size of the pericellular brush layer was quite expected between quiescent and proliferating phenotypes due to significant heterogeneity of cancer and inter-human variability. The observed similar trend for all patients for the grafting density of the pericellular brush (as well as the amount of the brush and the effective volume molecular density of the brush) is an interesting and unexpected observation. If statistically confirmed on a larger number of human subjects, this might be used as a new strategy to attack on this cancer. For example, it may be used to develop special drug carriers that will be attracted preferentially to

1
2
3
4 the denser brush of the quiescent leukemia stem cells. We also see the major significance of the
5
6 present results as a demonstration of the ability of the AFM technique to differentiate mechanics
7
8 of LSC cells and their pericellular coat for the purpose of possible personalization and future
9
10 development of treatment of this cancer.
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 **Acknowledgements:**
5

6
7 We are thankful to one of the reviewers for the idea of introducing the effective volume
8
9 molecular density parameter of the brush. B.D.C. is thiankful to Nancy Taylor, Judy Murray and
10
11 Alexander McLanahan.
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Reference

1. S. Faderl, M. Talpaz, Z. Estrov, S. O'Brien, R. Kurzrock and H. M. Kantarjian, The biology of chronic myeloid leukemia. *N Engl J Med*. 1999;**341**:164-72
2. B. Clarkson, A. Strife, D. Wisniewski, C. L. Lambek and C. Liu, Chronic myelogenous leukemia as a paradigm of early cancer and possible curative strategies. *Leukemia*. 2003;**17**:1211-62
3. E. Jabbour and H. Kantarjian, Chronic myeloid leukemia: 2012 update on diagnosis, monitoring, and management. *Am J Hematol*. 2012;**87**:1037-45
4. E. Jabbour and H. Kantarjian, Chronic myeloid leukemia: 2014 update on diagnosis, monitoring, and management. *Am J Hematol*. 2014;**89**:547-56
5. G. D. Miller, B. J. Bruno and C. S. Lim, Resistant mutations in CML and Ph(+)ALL - role of ponatinib. *Biologics*. 2014;**8**:243-54
6. J. R. Sierra, V. Cepero and S. Giordano, Molecular mechanisms of acquired resistance to tyrosine kinase targeted therapy. *Mol Cancer*. 2010;**9**:75
7. K. Sweet and V. Oehler, Discontinuation of tyrosine kinase inhibitors in chronic myeloid leukemia: when is this a safe option to consider? *Hematology Am Soc Hematol Educ Program*. 2013;**2013**:184-8
8. F. X. Mahon, Is going for cure in chronic myeloid leukemia possible and justifiable? *Hematology Am Soc Hematol Educ Program*. 2012;**2012**:122-8
9. H. Y. Yhim, N. R. Lee, E. K. Song, C. Y. Yim, S. Y. Jeon, B. Lee, et al., Long-Term Outcomes after Imatinib Mesylate Discontinuation in Chronic Myeloid Leukemia Patients with Undetectable Minimal Residual Disease. *Acta Haematol*. 2015;**135**:133-139

- 1
2
3
4 10. B. D. Clarkson, 1974. The Survival Value of the Dormant State in Neoplastic and Normal
5
6 Cell Populations in B. D. Clarkson and R. 8aserga (eds) *COLD SPRING HARBOR*
7
8 *CONFERENCES ON CELL PROLIFERATION*, Cold Spring Harbor Laboratory, Woodbury, NY
9
10 pp 945-972
11
12
13
14 11. C. R. Cogle, Overcoming chronic myeloid leukemia stem cell resistance to imatinib by
15
16 also targeting JAK2. *J Natl Cancer Inst.* 2013;**105**:378-9
17
18
19 12. I. Sokolov, 2007. Atomic Force Microscopy in Cancer Cell Research in H. S. N. a. T.
20
21 Webster (ed) *Cancer Nanotechnology – Nanomaterials for Cancer Diagnosis and Therapy*, APS,
22
23 Los Angeles pp 43-59
24
25
26 13. I. Sokolov, M. Firtel and G. S. Henderson, Insitu high-resolution AFM imaging of
27
28 biological surfaces. *Journal of Vac.Sci.&Tech. B.* 1996;**14**:674-678
29
30
31 14. H. I. Rasool, P. R. Wilkinson, A. Z. Stieg and J. K. Gimzewski, A low noise all-fiber
32
33 interferometer for high resolution frequency modulated atomic force microscopy imaging in
34
35 liquids. *Rev Sci Instrum.* 2010;**81**:-
36
37
38 15. M. E. Dokukin, N. V. Guz, R. M. Gaikwad, C. D. Woodworth and I. Sokolov, Cell
39
40 Surface as a Fractal: Normal and Cancerous Cervical Cells Demonstrate Different Fractal
41
42 Behavior of Surface Adhesion Maps at the Nanoscale. *Physical Review Letters.* 2011;**107**
43
44
45 16. J. Reed, C. Hsueh, B. Mishra and J. K. Gimzewski, Atomic force microscope observation
46
47 of branching in single transcript molecules derived from human cardiac muscle.
48
49
50 *Nanotechnology.* 2008;**19**:-
51
52
53 17. M. Dokukin and I. Sokolov, High-resolution high-speed dynamic mechanical
54
55 spectroscopy of cells and other soft materials with the help of atomic force microscopy.
56
57
58 *Scientific reports.* 2015;**5**
59
60
61
62
63
64
65

18. E. A-Hassan, W. F. Heinz, M. D. Antonik, N. P. D'Costa, S. Nageswaran, C. A. Schoenenberger, et al., Relative microelastic mapping of living cells by atomic force microscopy. *Biophysical journal*. 1998;**74**:1564-1578
19. T. K. Berdyeva, C. D. Woodworth and I. Sokolov, Human epithelial cells increase their rigidity with ageing in vitro: direct measurements. *Physics in Medicine and Biology*. 2005;**50**:81-92
20. S. Iyer, R. M. Gaikwad, V. Subba-Rao, C. D. Woodworth and I. Sokolov, AFM Detects Differences in the Surface Brush on Normal and Cancerous Cervical Cells. *Nature Nanotechnology*. 2009;**4**:389-393
21. S. Suresh, Biomechanics and biophysics of cancer cells. *Acta Mater*. 2007;**55**:3989-4014
22. M. E. Dokukin, N. V. Guz, S. Vasilyev and I. Sokolov, Atomic force microscopy to detect internal live processes in insects. *Applied Physics Letters*. 2010;**96**
23. S. E. Cross, Y. S. Jin, J. Rao and J. K. Gimzewski, Nanomechanical analysis of cells from cancer patients. *Nature Nanotechnology*. 2007;**2**:780-783
24. M. Lekka, D. Gil, K. Pogoda, J. Dulinska-Litewka, R. Jach, J. Gostek, et al., Cancer cell detection in tissue sections using AFM. *Arch Biochem Biophys*. 2012;**518**:151-156
25. W. R. Trickey, G. M. Lee and F. Guilak, Viscoelastic properties of chondrocytes from normal and osteoarthritic human cartilage. *J Orthopaed Res*. 2000;**18**:891-898
26. J. P. Shelby, J. White, K. Ganesan, P. K. Rathod and D. T. Chiu, A microfluidic model for single-cell capillary obstruction by Plasmodium falciparum infected erythrocytes. *P Natl Acad Sci USA*. 2003;**100**:14618-14622

- 1
2
3
4 27. N. Golenhofen, A. Redel, E. F. Wawrousek and D. Drenckhahn, Ischemia-induced
5 increase of stiffness of alpha B-crystallin/HSPB2-deficient myocardium. *Pflug Arch Eur J Phy.*
6
7 2006;**451**:518-525
8
9
10
11 28. M. Stolz, R. Gottardi, R. Raiteri, S. Miot, I. Martin, R. Imer, et al., Early detection of
12 aging cartilage and osteoarthritis in mice and patient samples using atomic force microscopy. *Nat*
13
14 *Nanotechnol.* 2009;**4**:186-92
15
16
17 29. J. T. Zahn, I. Louban, S. Jungbauer, M. Bissinger, D. Kaufmann, R. Kemkemer, et al.,
18 Age-Dependent Changes in Microscale Stiffness and Mechanoresponses of Cells. *Small.*
19
20 2011;**7**:1480-1487
21
22
23 30. N. Guz, M. Dokukin, V. Kalaparthi and I. Sokolov, If cell mechanics can be described by
24 elastic modulus: study of different models and probes used in indentation experiments. *Biophys*
25
26 *J.* 2014;**107**:564-75
27
28
29 31. A. Gruverman, O. Auciello and H. Tokumoto, Imaging and control of domain structures
30 in ferroelectric thin films via scanning force microscopy. *Annu. Rev. Mater. Sci.* 1998;**28**:101-
31
32 123
33
34 32. P. Carl and H. Schillers, Elasticity measurement of living cells with an atomic force
35 microscope: data acquisition and processing. *Pflug Arch Eur J Phy.* 2008;**457**:551-559
36
37
38 33. Z. Teff, Z. Priel and L. A. Gheber, Forces applied by cilia measured on explants from
39 mucociliary tissue. *Biophys J.* 2007;**92**:1813-23
40
41
42 34. I. Sokolov, S. Iyer, V. Subba-Rao, R. M. Gaikwad and C. D. Woodworth, Detection of
43 surface brush on biological cells in vitro with atomic force microscopy. *Applied Physics Letters.*
44
45 2007;**91**:023902-1-3
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1
2
3
4 35. J. D. Rowley, Letter: A new consistent chromosomal abnormality in chronic
5
6 myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature*.
7
8 1973;**243**:290-3
9
- 10
11 36. M. Affer, S. Dao, C. Liu, A. B. Olshen, Q. Mo, A. Viale, et al., Gene Expression
12
13 Differences between Enriched Normal and Chronic Myelogenous Leukemia Quiescent
14
15 Stem/Progenitor Cells and Correlations with Biological Abnormalities. *J Oncol*.
16
17 2011;**2011**:798592
18
19
20
- 21 37. D. E. Hogge, P. M. Lansdorp, D. Reid, B. Gerhard and C. J. Eaves, Enhanced detection,
22
23 maintenance, and differentiation of primitive human hematopoietic cells in cultures containing
24
25 murine fibroblasts engineered to produce human steel factor, interleukin-3, and granulocyte
26
27 colony-stimulating factor. *Blood*. 1996;**88**:3765-73
28
29
30
- 31 38. C. J. Hogan, E. J. Shpall and G. Keller, Differential long-term and multilineage
32
33 engraftment potential from subfractions of human CD34+ cord blood cells transplanted into
34
35 NOD/SCID mice. *Proc Natl Acad Sci U S A*. 2002;**99**:413-8
36
37
- 38 39. J. L. McKenzie, O. I. Gan, M. Doedens, J. C. Wang and J. E. Dick, Individual stem cells
39
40 with highly variable proliferation and self-renewal properties comprise the human hematopoietic
41
42 stem cell compartment. *Nat Immunol*. 2006;**7**:1225-33
43
44
- 45 40. A. Gothot, R. Pyatt, J. McMahon, S. Rice and E. F. Srouf, Functional heterogeneity of
46
47 human CD34(+) cells isolated in subcompartments of the G0 /G1 phase of the cell cycle. *Blood*.
48
49 1997;**90**:4384-93
50
51
52
- 53 41. N. Almqvist, R. Bhatia, G. Primbs, N. Desai, S. Banerjee and R. Lal, Elasticity and
54
55 adhesion force mapping reveals real-time clustering of growth factor receptors and associated
56
57 changes in local cellular rheological properties. *Biophysical Journal*. 2004;**86**:1753-1762
58
59
60
61
62
63
64
65

42. S. G. Shroff, D. R. Saner and R. Lal, Dynamic Micromechanical Properties of Cultured Rat Atrial Myocytes Measured by Atomic-Force Microscopy. *Am J Physiol-Cell Ph.* 1995;**269**:C286-C292
43. I. Sokolov, M. E. Dokukin and N. V. Guz, Method for quantitative measurements of the elastic modulus of biological cells in AFM indentation experiments. *Methods.* 2013;**60**:202-213
44. M. E. Dokukin, N. V. Guz and I. Sokolov, Quantitative Study of the Elastic Modulus of Loosely Attached Cells in AFM Indentation Experiments. *Biophys J.* 2013;**104**:2123-31
45. S. Nawaz, P. Sanchez, K. Bodensiek, S. Li, M. Simons and I. A. Schaap, Cell visco-elasticity measured with AFM and optical trapping at sub-micrometer deformations. *PLoS One.* 2012;**7**:e45297
46. M. Simon, M. Dokukin, V. Kalaparthi, E. Spedden, I. Sokolov and C. Staii, Load Rate and Temperature Dependent Mechanical Properties of the Cortical Neuron and Its Pericellular Layer Measured by Atomic Force Microscopy. *Langmuir.* 2016;**32**:1111-1119
47. J. Israelachivili, 2011. *Intermolecular and Surface Forces* Academic. Press, Burlington, MA.
48. M. Y. Gordon, S. B. Marley, J. F. Apperley, D. Marin, J. Kaeda, R. Szydlo, et al., Clinical heterogeneity in chronic myeloid leukaemia reflecting biological diversity in normal persons. *Br J Haematol.* 2003;**122**:424-9
49. A. Hernandez, L. Corral, A. Muniz, C. Alaez, E. Espinosa, G. Martinez, et al., Further evidence for the molecular heterogeneity of chronic myeloid leukemia. *Ann Hematol.* 1991;**62**:217-20
50. J. Sessions, Chronic myeloid leukemia in 2007. *Am J Health Syst Pharm.* 2007;**64**:S4-9
51. S. Suresh, Biomechanics and biophysics of cancer cells. *Acta Biomater.* 2007;**3**:413-38

- 1
2
3
4 52. R. Kurzrock, J. U. Gutterman and M. Talpaz, The molecular genetics of Philadelphia
5
6 chromosome-positive leukemias. *N Engl J Med.* 1988;**319**:990-8
7
8
9 53. C. Preisinger and W. Kolch, The Bcr-Abl kinase regulates the actin cytoskeleton via a
10
11 GADS/Slp-76/Nck1 adaptor protein pathway. *Cell Signal.* 2010;**22**:848-56
12
13
14 54. P. A. Ferreira, R. R. Ruela-de-Sousa, K. C. Queiroz, A. C. Souza, R. Milani, R. A. Pilli,
15
16 et al., Knocking down low molecular weight protein tyrosine phosphatase (LMW-PTP) reverts
17
18 chemoresistance through inactivation of Src and Bcr-Abl proteins. *PLoS One.* 2012;**7**:e44312
19
20
21 55. W. A. Lam, M. J. Rosenbluth and D. A. Fletcher, Chemotherapy exposure increases
22
23 leukemia cell stiffness. *Blood.* 2007;**109**:3505-8
24
25
26 56. M. Cohen, E. Klein, B. Geiger and L. Addadi, Organization and adhesive properties of
27
28 the hyaluronan pericellular coat of chondrocytes and epithelial cells. *Biophys J.* 2003;**85**:1996-
29
30 2005
31
32
33 57. L. M. Jones, M. J. Gardner, J. B. Catterall and G. A. Turner, Hyaluronic acid secreted by
34
35 mesothelial cells: a natural barrier to ovarian cancer cell adhesion. *Clinical & experimental*
36
37 *metastasis.* 1995;**13**:373-80
38
39
40 58. S. Kumar and J. H. Hoh, Modulation of repulsive forces between neurofilaments by
41
42 sidearm phosphorylation. *Biochemical and Biophysical Research Communications.*
43
44 2004;**324**:489-496
45
46
47 59. B. Toole, 1982. Glycosaminoglycans in morphogenesis in E. Hay (ed) *Cell Biology of the*
48
49 *Extracellular Matrix*, Plenum Press, New York pp 259-294
50
51
52 60. E. Zimmerman, B. Geiger and L. Addadi, Initial stages of cell-matrix adhesion can be
53
54 mediated and modulated by cell-surface hyaluronan. *Biophysical journal.* 2002;**82**:1848-57
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure Captions

Figure 1. A schematic of interaction of a spherical AFM probe with a cell loosely attached to the bottom of a culture dish used in the described model. The cell body treated as a homogeneous elastic material is surrounded by the pericellular (brush) layer. Note that the assumption of entropic behavior of the brush layer is required only for extraction of the brush layer parameters, the equilibrium length L and grafting density N .

Figure 2. An example of processing of the raw AFM curve (Z vs d) recorded on the surface of the quiescent CML stem cells through the brush model. (a, c) fitting of the different regions of raw Z - d curve with Eq.(2). (b) Forces of interaction between the AFM probe and the brush layer obtained by processing raw Z - d curve for the $d=55$ - 90 nm (here open circles correspond to the force-separation data, solid line is the model fitting Eq(4)).(d) the best Hertz fitting for the entire range of the nonzero force. Deviation of the model curves from the experimental data demonstrates the limit of applicability of the described brush model and the reference Hertz model.

Figure 3. Quantitative results for cells collected from four patients. Both G_0 and $G1$ phases are shown. The average and SEM values are plotted for (a) the Young's moduli, (b) brush length, (c) grafting density, (d) the total size brush ($N*L$ parameter), (e) and effective molecular volume density (N/L parameter).

Tables

Patients	CD34+ G ₀ Cells			CD34+ (G1/S/G2/M) Cells		
	No. of cells	Total force curves	No. of force curve per cell	No. of cells	Total force curves	No. of force curve per cell
1	6	58	5-18	2	41	22 & 19
2	3	31	9-12	6	70	8-15
3	15	126	5-12	13	105	6-9
4	10	92	8-13	12	97	5-11

Table 1. Number of CML CD34+ G₀ and CD34+ G1/S/G2/M cells studied using AFM. It also shows total number of force curves acquired and average number of force curves per cell.

	patient 1 G0	patient 1 G1	patient 2 G0	patient 2 G1	patient 3 G0	patient 3 G1	patient 4 G0	patient 4 G1
moduli [kPa]	7.1±0.5	4.0±0.3	5.0±0.7	5.0±0.4	6.4±0.3	8.8±0.4	4.1±0.2	5.5±0.3
L [nm]	416±58	860±134	600±87	690±120	1055±85	765±60	525±78	562±64
N [um ⁻²]	370±54	70±12	260±35	74±12	650±58	500±40	310±48	280±37
L*N ×10 ³ [um ⁻¹]	300±51	126±22	157±18	106±16	621±42	533±64	204±30	172±19
N/L [um ⁻³]	2030±440	290±20	1200±250	400±240	1990±280	1320±170	2350±440	2010±360

Table 2. Averaged and SEM values of the Young's modulus, the brush length L, grafting density N, and the brush size (N*L), and and effective molecular volume density (N/L) parameters.

Figure1

Figure2a

Figure2b

Figure2c

Figure2d

Figure3a

Figure3b

Figure3c

Figure3d

Figure3e

GRAPHICAL ABSTRACT:

Atomic force microscopy (AFM) was used here to study chronic myeloid leukemia (CML) stem cells in dormant (quiescent) and proliferating stages. Persistence of residual *quiescent* CML stem cells (LSC) that later resume proliferation seems to be a common cause of recurrence or relapse of CML. The collage shows a scheme of the AFM method and the found biophysical difference between these cells in the density of the pericellular layer surrounding cells.