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Quest for Pan-Cancer Diagnosis/Prognosis Ends with HrC Test Measuring Oct4A in Peripheral Blood

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Abstract

Cancer is a devastating disease whose incidence has increased in recent times and early detection can lead to effective treatment. Existing detection tools suffer from low sensitivity and specificity, and are high cost, invasive and painful procedures. Cancers affecting different tissues, ubiquitously express embryonic markers including Oct-4A, whose expression levels have also been correlated to staging different types of cancer. Cancer stem cells (CSCs) that initiate cancer are possibly the 'transformed' and pluripotent very small embryonic-like stem cells (VSELs) that also express OCT-4A. Excessive self-renewal of otherwise quiescent, pluripotent VSELs in normal tissues possibly initiates cancer. In an initial study on 120 known cancer patients, it was observed that Oct-4A expression in peripheral blood correlated well with the stage of cancer. Based on these results, we developed a proprietary HrC scale wherein fold change of OCT-4A was linked to patient status – it is a numerical scoring system ranging from non-cancer (0–2), inflammation (>2–6), high-risk (>6–10), stage I (>10–20), stage II (>20–30), stage III (>30–40), and stage IV (>40) cancers. Later the scale was validated on 1000 subjects including 500 non-cancer and 500 cancer patients. Ten case studies are described and show (i) HrC scale can detect cancer, predict and monitor treatment outcome (ii) is superior to evaluating circulating tumor cells and (iii) can also serve as an early biomarker. HrC method is a novel breakthrough, non-invasive, blood-based diagnostic tool that can detect as well as classify solid tumors, hematological malignancies and sarcomas, based on their stage.

Keywords Cancer diagnosis · HrC · VSELs · Oct4A.

Introduction

Patients, clinicians and the scientific community have been fighting cancer for decades but it still remains to be understood how cancer initiates and why it recurs –and what leads to its heterogeneity, tumorigenicity, progression and metastasis. Quiescent cancer stem cells (CSCs) which are resistant to oncotherapy were proposed to exist almost 4 decades

Highlights
- Cancers ubiquitously express embryonic marker Oct-4A, whose expression levels are related to stages of cancer.
- Cancer is initiated by the excessive self-renewal of very small embryonic-like stem cells (VSELs) that express Oct-4A.
- We developed a proprietary HrC scale wherein fold change of OCT-4A was linked to patient status.
- HrC scale can effectively screen, diagnose and prognose cancer with 100% sensitivity and specificity.

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ago [1]. However, CSCs still remain controversial as it has proved difficult to isolate them [2]. More than thirty independent groups have now confirmed the presence of pluripotent stem cells termed very small embryonic-like stem cells (VSELs) in adult tissues [3]. These are the most primitive and pluripotent stem cells that survive throughout life in multiple tissues, are quiescent in nature and undergo asymmetrical cell divisions whereby they self-renew and give rise to tissue-specific progenitors that further differentiate into tissue-specific cell types [3, 4]. Ratajczak's group discussed that misappropriated differentiation of VSELs possibly leads to cancer initiation [5]. They showed an increased mobilization of VSELs in the peripheral blood in mice injected with C2C12 cancer cells [6] and were the first group to propose a possible role of VSELs in initiating cancer [7].

Being pluripotent, VSELs express OCT-4, ALDH and CD133 which are also expressed by cancers affecting several tissue types. Bhartiya's group provided evidence that the dysfunction of VSELs due to neonatal exposure of mice pups to endocrine disruption initiates testicular cancer in adult mice [8]. More than 7 folds increase in VSEL numbers was observed by flow cytometry along with and their blocked differentiation that led to various pathologies. Earlier, Virant-Klun's group has reported increased numbers of VSELs in clinical samples of ovarian cancer [9]. VSELs get mobilized under stress conditions and similarly CSCs show potential to metastasize distant sites. Both VSELs in normal tissues and CSCs in cancer tissues exist in few numbers and are quiescent in nature. In the present study we studied OCT-4 expression in the VSELs in the peripheral blood and made an attempt to correlate it with cancer stage. Samardzija and co-workers reported a crucial role of Oct4A in the progression and metastasis of epithelial ovarian tumors [10] and further suggested that targeting Oct4A may prove to be an effective strategy in the treatment and management of epithelial ovarian tumors. Based on a meta-analysis, Zhao et al. [11] reported that OCT-4 expression in solid tumors is a potential biomarker for solid tumors. VSELs express nuclear OCT-4A which reflects their pluripotent state. Cytoplasmic OCT-4B is expressed in the immediate descendants of tissue specific progenitors. Since VSELs are located in all adult tissues, Oct-4A expression will detect all kinds of tumors not limited to only germ cell tumors. In the present study we studied OCT-4 expression in the VSELs in the peripheral blood and made an attempt to correlate it with cancer stage. It needs to be appreciated that VSELs in normal tissues are distinctly different from those in cancer tissues where they have most likely transformed into cancer stem cells (CSCs). VSELs in normal tissues remain quiescent and maintain tissue homeostasis in a very subtle manner. Whereas, CSCs (transformed VSELs) are increased in numbers in cancer samples and their further differentiation gets blocked. This leads to selective enrichment of Oct-4A in cancer tissues and was studied in the present study.

Rather than studying OCT-4 expression (including OCT-4A and other isoforms), we hypothesized that OCT-4A expression (specific for VSELs) in peripheral blood will predict all kinds of cancers and possibly their stage because of the ubiquitous presence of VSELs in multiple adult tissues. The present multicenter, investigator-blinded pilot clinical study was undertaken to study the possible use of OCT-4A transcript in peripheral blood as a non-invasive bio-marker to detect & stage cancer and monitor the effect of oncotherapy. Transformed VSELs (CSCs) were enriched from the peripheral blood and studied for the expression of *OCT-4A* by qRT-PCR and correlated with the absence, presence and cancer stage of the patients.

Materials and Methods

Study Design

The study was sponsored by Epigeneres Biotech Pvt. Ltd., Mumbai, India after taking ethics approval from Ethics Committee of Maharashtra Technical Education Society at Sanjeevan Hospital, Pune, India and was registered with Clinical Trial Registry India (CTRI/2019/01/017166).

Initially 120 samples were collected along with patient history and all the related information. Oct-4A mRNA expression was studied in the VSELs enriched from peripheral blood which helped arrive at a scale (HrC scale) wherein cancer stage was correlated to fold change in OCT-4A expression. Once the scale was obtained, its validation was done in a total of 1000 subjects which were recruited from seven different sites for the study, of whom 500 were non-cancer and 500 were cancer patients (Table 1). The samples were blinded by PA and the analysis of blinded samples was conducted by SC, AT, and VT. The patients with histologically or cytologically proven malignancy, either solid tumors or hematological malignancy, were included in the cancer group after obtaining written informed consent. As per medical history, out of 500

Table 1 Subject details

Patient population	Number of participants
Gender	
Male	534
Female	466
Age (years)	
Mean	61.3
Weight (kg)	
Mean	69.3
Height (cm)	
Mean	161.38

cancer subjects, 100 had co-morbidities and out of 500 non-cancer subjects, 76 had co-morbidities. Circulating tumor cells (CTCs) were studied randomly in a few cases of interest.

Blood Sample Processing

Blood samples (approximately 10 ml) were collected from the subjects and processed to enrich VSELs as described earlier [12]. Briefly the samples were layered over Ficoll-Hypaque and subjected to density gradient centrifugation at 1200 rpm for 15 min. Post centrifugation, cells in the RBCs fraction were subjected to lysis and then centrifuged at 3000 rpm (1000 g) to pellet down all the cell types including the granulocytes and VSELs. These cells were then centrifuged at 250 g that allowed granulocytes to settle down. The supernatant was further centrifuged at 1000 g when VSELs (remain buoyant at 250 g) settled down. Cell smears were prepared of the enriched population of VSELs. For this the cells were fixed with 4% paraformaldehyde, then washed with PBS and placed on a slide. After air drying, the cells were stained with Hematoxylin and Eosin and viewed under a microscope to take representative images. While processing, stem cells were always centrifuged at 1000 g. As discussed earlier [13], this high speed does not affect the stem cells as they have minimal cytoplasm and cytoplasmic organelles compared to the mature somatic cells. 2 ml of peripheral blood from normal and cancer patients were separately processed using similar protocol and suspended in the same volume of PBS for making smears. This method allowed enrichment of VSELs since mononuclear cells in the buffy coat were discarded upon Ficoll-Hypaque centrifugation and were not used for the study. Study was carried out on the VSELs that pellet down with the RBCs as reported earlier [12].

RNA Isolation and cDNA Synthesis

Total RNA was extracted from the VSELs pellet using RNAplus (MP Biomedicals, Irvine, USA) according to manufacturer's instructions. After RNA extraction, first-strand cDNA was synthesized using the Revert Aid First strand cDNA synthesis kit (Thermo scientific, UK) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was incubated with 5X Reaction Buffer and reverse transcriptase mix. The reaction was carried out in Applied Biosystems GeneAmp® thermal cycler 9700 (Applied Biosystems, USA) as per manufacturer's instructions.

qRT-PCR Studies

The expression level of Oct4A gene transcript was estimated by real-time PCR system-ABI 7500 (Applied Biosystems, USA) using Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix kit (Thermo scientific, UK) and gene specific primer sequences (Oct4A: Forward AGCCCTCATTTCACCAGGCC Reverse TGGGACTCCTCCGGGTTTTG). The 18 s rRNA gene was used as a housekeeping gene. The amplification conditions were: initial denaturation at 94 °C for 3 min followed by 45 cycles comprising denaturation at 94 °C for 30 s, primer annealing at 62 °C for 30 s, and extension at 72 °C for 30 s followed by melt curve analysis step from 55 °C to 95 °C. The fluorescence emitted was collected during the extension step of each cycle. The homogeneity of the PCR amplicons was verified by studying the melt curve. C_t values generated in each experiment using the 7500 Manager software (Applied Bio-systems, UK) were used to calculate the mRNA expression levels.

Estimating Circulating Tumor Cells (CTCs) in Blood Plasma Samples

CTCs are found in patients with solid tumors and function as seeds for metastasis [14]. They are considered clinical biomarkers and therapeutic targets and are considered a component of liquid biopsy. CTCs were estimated using an earlier published protocol. 10 ml of blood sample, collected in EDTA tubes, was subjected to centrifugation at 820 g for 10 min. Supernatant was transferred to another tube and centrifuged at 16,000 g for 10 min to pellet any remaining cellular debris and then stored at −80 °C. Total genomic DNA was extracted from 2 ml of plasma samples using QIAamp MinElute kit (Qiagen) according to the manufacturer's instructions. The amount of total DNA isolated from plasma was quantified with Nanodrop and was analyzed for the expression of the human LINE-1 gene using quantitative real-time PCR as described previously [15]. Three primer sets were used to amplify differently sized regions within the most abundant consensus region of the human LINE-1 family (79 bp forward: 5'-AGGGACATGGATGAAATTGG-3′. 79 bp reverse: 5′-TGAGAATATGCGGTGTTTGG-3′; 97 bp forward: 5'-TGGCACATATACACCATGGAA-3′, 97 bp reverse: 5′-TGAGAATGATGGTTTCCAATTTC-3′; 127 bp forward: 5'-ACTTGGAACCAACCCAAATG-3′, 127 bp reverse: 5′- TCATCCATGTCCCTACAAAGG-3′). PCR was performed in a 25 μl reaction volume consisting of template DNA equal to 2 μl of plasma, 0.5 U of Taq DNA Polymerase, 1× PCR buffer, 6% (*v*/v) DMSO, 1 mM of each dNTP, 5 μl of SYBR Green and 0.2 μM of each primer. Amplification was carried out in Cycler using the following cycling conditions: 94 °C for 1 min; 2 cycles of 94 °C for 10 s, 67 °C for 15 s, 70 °C for 15 s; 2 cycles of 94 °C for 10 s, 64 °C for 15 s, 70 °C for 15 s, 2 cycles of 94 °C for 10 s, 61 °C for 15 s, 70 °C for 15 s; 35 cycles of 94 °C for 10 s, 59 °C for 15 s, 70 °C for 15 s.

Results

Cell Smears

Small, distinctly spherical cells with high nucleo-cytoplasmic ratio and darkly stained nuclei were clearly observed in smears from both normal (Fig. 1a-b) and cancer (Fig. 1c-d) patient (stage 3). Evidently the numbers of VSELs were greatly increased in the cancer patient compared to the normal sample.

Developing the HrC Scale

Initially a HrC scale was developed based on Oct-4A expression in 120 samples. The Oct4A expression in peripheral blood was correlated with the medical history (PET scan and biopsy reports). It was observed that Oct4A was manifold upregulated in peripheral blood of cancer patients when compared to non-cancer subjects. Within cancer patients, the expression of OCT4A was highest for stage 4 cancer and lowest for stage 1. On the basis of fold increase, we formulated a HrC scale using which we could segregate non-cancer and cancer subjects and also stage the cancer. The HrC scale is calculated based on ratio of expression levels of Oct4A gene in PB enriched VSELs to housekeeping gene and standardized the ratio based on a scaling factor. The non-cancer patients and those with increased inflammation that could lead to cancer initiation (on correlating with patient history) in future also revealed a specific range of values. The subjects were identified and distributed on the basis of their HrC score as non-cancer, inflammation, high risk, stage I cancer, stage II cancer, stage III cancer and stage IV cancer (Fig. 2).

Validation of HrC Scale

Between January 2019 and May 2019, a total of 1051 patients were screened and recruited for the study. 51 of 1051 subjects were excluded because of screen failures. There were 534 males and 466 females. The median patient age was 63.0 years for the complete dataset. The mean weight was 69.3 kg and mean height was 161.38. Table 1 summarizes patient demographics for the complete dataset.

Of the 500 cancer patients, 431 patients were on treatment (R_x), 48 were not subjected to any treatment after diagnosis of cancer (R_x Naïve) and 21 patients had undergone surgical intervention for cancer treatment (R_0). Patients with 25 different types of cancers were included in the study as shown in Fig. 3.

Of the 1000 samples analyzed for HrC, 498 samples were non-cancerous, 7 were assessed to be in high risk stage, 11 were in stage I cancer, 94 were in stage II cancer, 133 were in stage III cancer, and 257 were in stage IV cancer (Fig. 4).

Table 2 describes ten cases in brief in which the HrC scale analysis proved useful for the cancer patients. Monitoring OCT-4A expression on the HrC scale was helpful in early prediction of cancer in a few cases leading to better management of patients, monitoring the effect of treatment, and it was more sensitive when compared to estimating CTCs. HrC levels were able to detect the presence of several types of solid

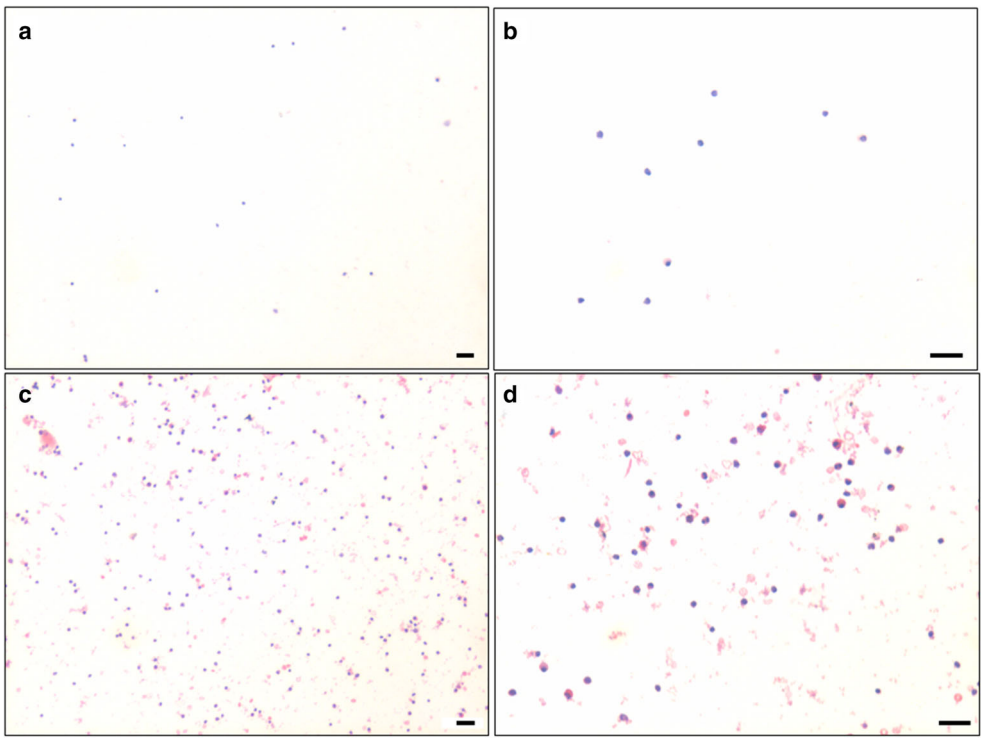

Fig. 1 H&E stained stem cells smears enriched from peripheral blood of a normal (**a**-**b**) and cancer patient (**c**-**d**). Note increased numbers of cancer stem cells (CSCs, transformed VSELs) in cancer patient compared to normal individual. Scale: 20X

Non-Cancer	0-2
Organ Inflammation	2-6
High Risk	6-10
Stage I Cancer	10-20
Stage II Cancer	20-30
Stage III Cancer	30-40
Stage IV Cancer	40-50

Fig. 2 HrC scale showing different ranges which were found to correlate with different stages of cancer

and liquid cancers. Figures 6, 7, 8 and 9 provide details of the results in all the 1000 study subjects. As evident, there was no ambiguity in the HrC values. To conclude, we describe a non-invasive test which can predict, screen and diagnose cancer with absolute specificity and sensitivity.

Validation of HrC Scale

To validate the HrC scale, the top 10 cancer types in terms of number of samples were chosen and the HrC values of the patients for each cancer type were plotted along with non-cancer healthy volunteers. As shown in Fig. 5, median HrC values of cancer patients were significantly higher as compared to non-cancer patients, implying validation of HrC scale to detect cancer.

Below figure provides details of the results in all the 1000 study subjects. As evident, there was no ambiguity in the HrC values. As shown in Fig. 5c, there is a significant transition in HrC values that differentiate between non-cancer and a variety of cancer types. To conclude, we describe a non-invasive test which can predict, screen and diagnose cancer with absolute (> 99%) specificity and sensitivity.

Outcome of HrC Diagnostic Test

As shown in Table S1 and Eqs. 1–3 of supplementary information, the sensitivity, specificity and accuracy of HrC test is 100% based on a 498 non-cancer, and 495 cancer data inclusion. 7 subjects out of 1000 were identified as high-risk and hence were not included in the above analysis since they were classified as neither cancer or non-cancer.

Performance of HrC Scale

HrC scale helped to screen, diagnose and prognose cancer with a sensitivity and specificity of 100%(495/495) and 100%(498/498) respectively (Table S2). We used R program to build a logistic regression model to understand the

Types Of Cancer	Number	
Breast Cancer	59	11.8%
Liver Cancer*	53	10.6%
Ovarian Cancer	48	9.6%
Lung Cancer	41	8.2%
Leukemia	38	7.6%
Renal Cancer	31	6.2%
Bladder Cancer	29	5.8%
Prostate Cancer	23	4.6%
Lymphoma Cancer	22	4.4%
Pancreatic Cancer	21	4.2%
Cervical Cancer	19	3.8%
Colon Cancer	17	3.4%
Osteosarcoma	16	3.2%
Testicular Cancer	15	3%
Thyroid Cancer	13	2.6%
Bile Duct Cancer	11	2.2%
Esophageal Cancer	11	2.2%
Endometrium	9	1.8%
Buccal Mucosa Cancer	5	1%
Gastric Cancer	5	1%
Tongue Cancer	5	1%
Retina Cancer	3	0.6%
Penile Cancer	3	0.6%
Ewing Sarcoma	2	0.4%
Gastrointestinal Stromal Tumor(GIST)	1	0.2%
Total	500	

*including liver metastases

Fig. 3 Distribution of types of cancer patients enrolled in the study

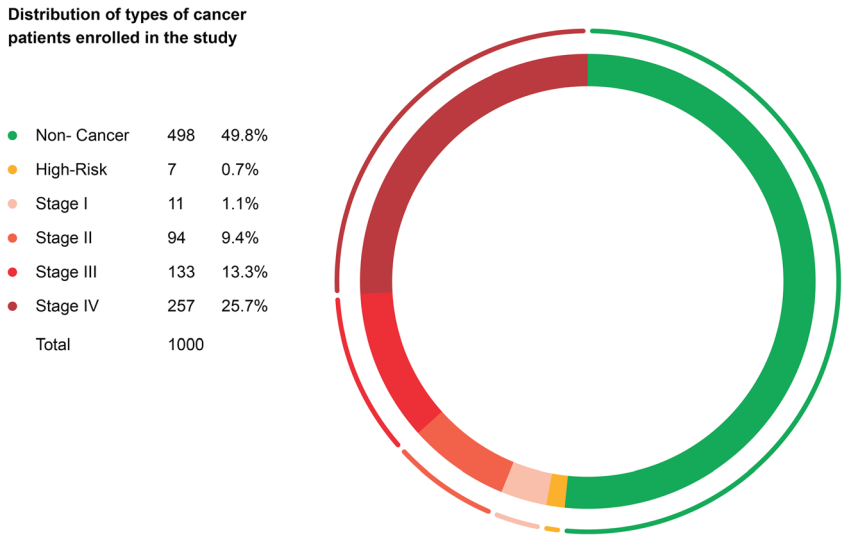

Fig. 4 Pie chart showing distribution of subjects identified as non-cancer (green), inflammation & high risk (dark yellow), Stage I cancer (pink), stage II cancer (red), stage III cancer (red) and stage IV cancer(purple) on the basis of their HrC score. High-risk subjects were determined based on HrC score between 6 and 10. We identified 7 high-risk individuals, some of which were prospectively followed until development of cancer and this has been described in detail in Table 2

prediction ability of our HrC scale. The model built is based on the 50% probability had a classification & accuracy rate of 100% between cancer & non cancer. The performance of this model was in turn evaluated using ROC curve and AUC(area under curve) using R package(ROCR). ROC curve is useful in the early stages of evaluation of a new diagnostic test and AUC is an effective way to summarize the overall diagnostic accuracy of the test. The ROC curve was generated using the logistic regression model by plotting the true positive rate(Sensitivity) on y axis against the false positive rate(1-Specificity) on X-axis (Fig. S1). The AUC for the model built is 1. There is no loss of diagnostic accuracy with 100% specificity, sensitivity based on age and gender interactions (data not shown). The diagnostic accuracy, sensitivity and specificity of Oct-4A expressing cells is 100% for solid cancers ($n = 457$). For haematological cancers ($n = 38$), the diagnostic accuracy is 100% (not shown).

Discussion

The study describes successful use of OCT-4A expression in the CSCs (transformed VSELs) enriched from the peripheral blood to monitor the cancer state of patients. CSCs (transformed VSELs) were greatly increased in numbers in cancer patients and Oct-4A expression in peripheral blood samples was useful to develop a HrC scale. This scale was extremely useful to screen, identify and stage cancer in high-risk clinical cases. Results of the present study suggest that the test is highly sensitive, there is no overlap and it can help monitor both disease progression as well as effect of therapy. Detection of a particular stage of cancer (I, II, III or IV) can assist doctors in decision making for stage-specific therapeutic treatment modalities, imminent cancer detection, and can lead to preventive strategies while the HrC scale testing after oncotherapy can help determine the effect of treatment and probability of recurrence. HrC values, along with NGS data on the VSELs enriched from the peripheral blood, could help provide personalized medicine to the patients in future.

The presence of VSELs in adult tissues is not yet widely accepted by the scientific community and recent single cell RNAseq studies failed to detect stem cells in adult tissues including ovary [16] and pancreas [17]. But VSELs do exist in ovaries and prostate and as reported by Bhartiya's group [18], the underlying reason for the controversy surrounding their existence is because of their very small size and being scarce in nature. The scientific community tends to process the small sized (2–6 μm) VSELs and the bigger (>8–10 μm), somatic mature cells with abundant cytoplasm together and inadvertently discards VSELs while processing for different experiments. A simple and robust protocol to enrich VSELs from multiple tissues including testes, uterus, pancreas was recently reported by Bhartiya's group [12, 18–20]. Cells suspension is first centrifuged at 250-350 g when the somatic cells pellet down (but VSELs remain buoyant) and later the VSELs can be enriched by centrifuging the supernatant at 1000 g. Once enriched, VSELs have been characterized in detail by various methods. VSELs are studied by flow cytometry as LIN-CD45-SCA-1+ in mouse tissues [5, 12]. They express pluripotent markers and also differentiate into 3 germ layers [21] justifying their pluripotent state but being quiescent in nature, VSELs do not form teratoma nor divide rapidly in vitro like embryonic stem cells. The underlying reasons for quiescence have been studied [22]. Keeping this basic understanding in mind, VSELs were enriched for the present study from the peripheral blood by centrifuging at 1000 g. VSELs exist in all adult tissues, and increase in numbers to initiate all kinds of cancers not limited to only the germ cell tumors. VSELs in normal tissues get transformed into CSCs and present study takes credit of developing a robust method to enrich

Table 2 Interesting cases highlighting the usefulness of HrC Scale developed in the present study

Case	Patient details	HrC levels	Additional Remarks
1	68-year-old male patient with liver cancer.	HrC value was 40.15 indicative of 4th stage liver cancer.	HrC test was able to accurately detect stage of cancer. Primary site of cancer was studied by analyzing the mutation and expression profile at transcriptome level by NGS of VSELs. A detailed mutational analysis revealed lymph node metastasis as per TNM classification. Further mutation analysis revealed primary and secondary organs like liver and lung with osseous metastasis. Cholangiocarcinoma was identified as the specific type of cancer and further sub-localization was identified using pathway analysis (data not shown).
2	55-year-old female was recruited in the cancer group with stage III cervicaladeno-carcinoma.	On the day of surgery, HrC value was 32.11. Four weeks post removal of the tumor surgically, HrC value fell to 9.14 which suggested that the patient still fell in the high risk category and required adjuvant chemotherapy. She underwent four cycles of adjuvant chemotherapy (Day 28 to Day 160) and PET scan was performed at the end which showed absence of lesion. The patient was declared cancer-free, and the HrC value showed a reading of 1.9 (Day 167) in alignment with PET scan.	The HrC test successfully aided oncologists to monitor the disease progression and risk of relapse.
3	68-year-old, 76-Kg male was recruited in the non-cancer group.	HrC value was 9.78 which suggested that he was at high risk of developing cancer. Based on HrC value, he was investigated in detail and enlargement of prostate was detected with PSA level 182 ng/ml and blood sugar levels 210 mg/dL and 170 mg/dL fasting and post prandial respectively. All other blood tests including complete blood count, blood urea nitrogen test, serum uric acid, liver function test and lipid test were normal. PET scan showed no signs of lesion across the body. After obtaining patient consent, radical prostatectomy was performed. On the day of surgery (9 weeks after first HrC analysis), HrC value had increased to 10.89 from 9.78. Four weeks post prostatectomy, HrC value was reduced to 2.1 which indicated slight organ inflammation.	The subject was experiencing generalized body weakness, acidity, abdominal pain and a weight loss. Patient management was carried out efficiently based on HrC test results The NGS analysis report on VSELs showed HoxB13 mutation and high grade*prostatic intra-epithelial neoplasia*-which is a precursor to prostate cancer.
4	65-year-old female with a tumor above ovary.	HrC value was 41.28 (Day 0) and cancer antigen 125 (CA-125) was 198.8 along with pain in abdomen (Day 3). Patient underwent surgery (Day 7) for the removal of both ovaries, uterus, and fallopian tube. Immuno-histochemistry analysis (Day 10) of the removed tissue suggested primary site of cancer being the stomach since the tissue was positive for CK-20 & CDX2/SATB2.	The doctors were unable to detect primary site of cancer and it was impacting the course of treatment (Day 30). Primary site of cancer was detected as the appendix by analyzing the mutation and expression profile at transcriptome level by Next Generation Sequencing on Day 44. *Rather than performing NGS on RNA extracted from total cells, RNA extracted from the VSELs (which comprise less than 1% of total cells) is more informative and can provide precision medicine to cancer patients.*
5	52-year-old male non-cancer subject.	The subject was recruited in the Non-cancer group, upon analysis it was found that HrC score was 7.86 indicating “high risk cancer” category.	HrC test was able to detect high risk category. Further analysis of mutation and expression profile at transcriptome level by Next Generation Sequencing revealed thyroid to be at risk of developing cancer. On further consultation with the oncologist, the subject underwent biopsy which revealed benign hemorrhagic nodules with degenerative changes and had extremely high levels (224 pg/ml) of

Table 2 (continued)

Case	Patient details	HrC levels	Additional Remarks
			calcitonin. The subject underwent total thyroidectomy.
6	68-year-old female with colorectal cancer was subjected to HrC and CTCs analysis.	HrC analysis revealed a score of 26.82 indicating stage II cancer. ctDNA were not detected in the blood.	Several researchers have reported non-reliability of ctDNA for accurate diagnosis of cancer (28).
7	47-year-old stage 3 breast cancer patient with a 7.4 cm left breast mass.	HrC value was 36.18 which classified her as Stage III. CTCs were not detected in blood by doing PCR for LINE1 on DNA extracted from the plasma samples. Core biopsy of the mass showed metastatic breast cancer, estrogen receptor (ER) 95% positive, progesterone receptor 85% positive and HER2 negative.	ASCO tumor marker guidelines (2007) suggest that measurement of CTCs should not be used for diagnosis or treatment modifications.
8	49-year-old male non-cancer subject.	The subject was recruited in the Non-cancer group, upon analysis it was found that HrC score was 7.20 indicating "high risk cancer" category.	HrC test was able to screen subjects at high risk for developing cancer. The patient was an active user of pan masala and gutkha and a regular smoker. In-depth analysis revealed that the subject was at risk of developing oral cancer.
9	78-year-old patient with carcinoma of lung & parenchymal non-cerebral metastasis.	The subject was recruited in the cancer group and HrC analysis showed value of 46.34 (indicating stage IV cancer on Day 0). Post two cycles of chemotherapy (Day 36) a diagnostic test was conducted again to assess the efficacy of the treatment. The HrC test at Day 36 showed a value of 42.38, clearly indicating effective therapy.	HrC test was used to assess the efficacy of the chemotherapy.
10	74-year-old male patient with Stage 4 liver cancer.	HrC test prior to neo-adjuvant chemotherapy was 49.43 (indicating stage 4 cancer patient). After three cycles of neo-adjuvant chemotherapy, HrC value dropped to 42.01.	HrC test can serve as a reliable marker for oncologists to interpret disease progression and effectiveness of the treatment. Patient was later lost on follow up.

CSCs and study them to monitor cancer stage for the first time.

It has been earlier reported that VSELs get enriched in the RBCs pellet and have been characterized in detail [12]. Cells in the 'buffy coat' are enriched for Hematopoietic stem cells and have been used globally for cell therapy. Mononuclear cells supposedly enriched for HSCs in the 'buffy coat' have failed to transdifferentiate and regenerate adult tissues as they are tissue-resident, lineage committed progenitors. The most primitive VSELs get separated with the RBCs and being pluripotent, are expected to have therapeutic potential [23]. VSELs get transformed into CSCs and increase in numbers in all types of leukemia/cancer. VSELs/CSCs are easily mobilized into circulation and their numbers may vary depending on the disease state of the patient. Thus, the HrC scale developed in the present study along with the clinical history will prove to be a useful approach for cancer diagnosis.

Existing "liquid biopsy" diagnostic tools are limited by their sensitivity and specificity, possibly because they utilize circulating tumor cells, cell free DNA etc., and a diverse set of biomarkers or DNA methylation profiles are investigated. Current diagnostic methods include PET CT scan, MRI and the gold standard of all methods, the tissue biopsy. Biopsy is expensive, invasive and painful, causes discomfort and the surgical procedures warrant undue, resultant side-effects [24]. Furthermore, due to inconspicuous anatomical locations, some tumor specimens are difficult to isolate making them inaccessible [25]. Also, tissue biopsies might not give accurate information due to tumor heterogeneity in gene expression and mutations. Tissue biopsies may augment risk of metastatic lesions and safety is also a concern, for e.g., related to sampling of angiogenic tumor microenvironments [26]. Similarly, imaging methods do not, at times, detect the cancer source, i.e., cancer of unknown primary (CUP) origin [27] is relatively frequent leading to inaccurate diagnosis affecting interventional therapies.

Various methods have been developed to diagnose cancer. However, one method that is prevalent in diagnostics is liquid biopsy mediated identification of cancer-specific signatures [28]. These signatures can be organ-specific gene expressions, tumor suppressor genes, oncogenic biomarkers, mutations or even DNA methylation markers that capture the presence of cancer in the body. Circulating tumor cells (CTCs), cancer stem cells (CSCs), hematopoietic stem cells etc., which are

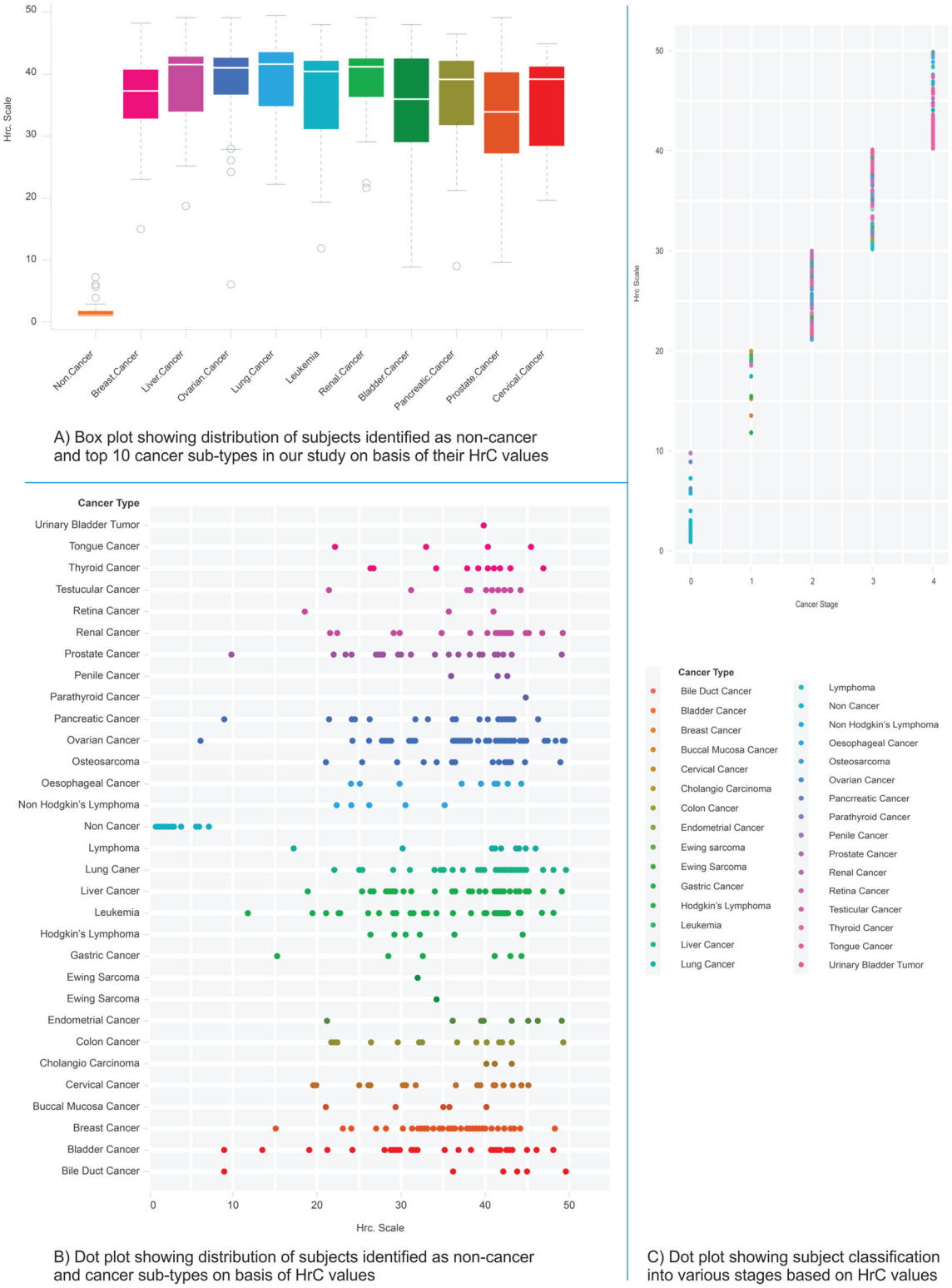

Fig. 5 Performance assessment of HrC test based on statistical analysis. Dot plot values correspond to 1000 patient sample points as per data of clinical study participants. All the figures were plotted using R package via ggplot library

present in whole blood, represent sources of tissue biomarkers for detecting cancer in vitro. However, while CTCs are rare and are detected in later stages of cancer, CSCs are also relatively rare, difficult to isolate and controversial in existence in the cancerous tissue as well as in whole blood samples. Lot of promise exists with the use of exosomes for cancer screening. Exosomes are extracellular vesicles that serve as biological messengers between cancer cells and express surface

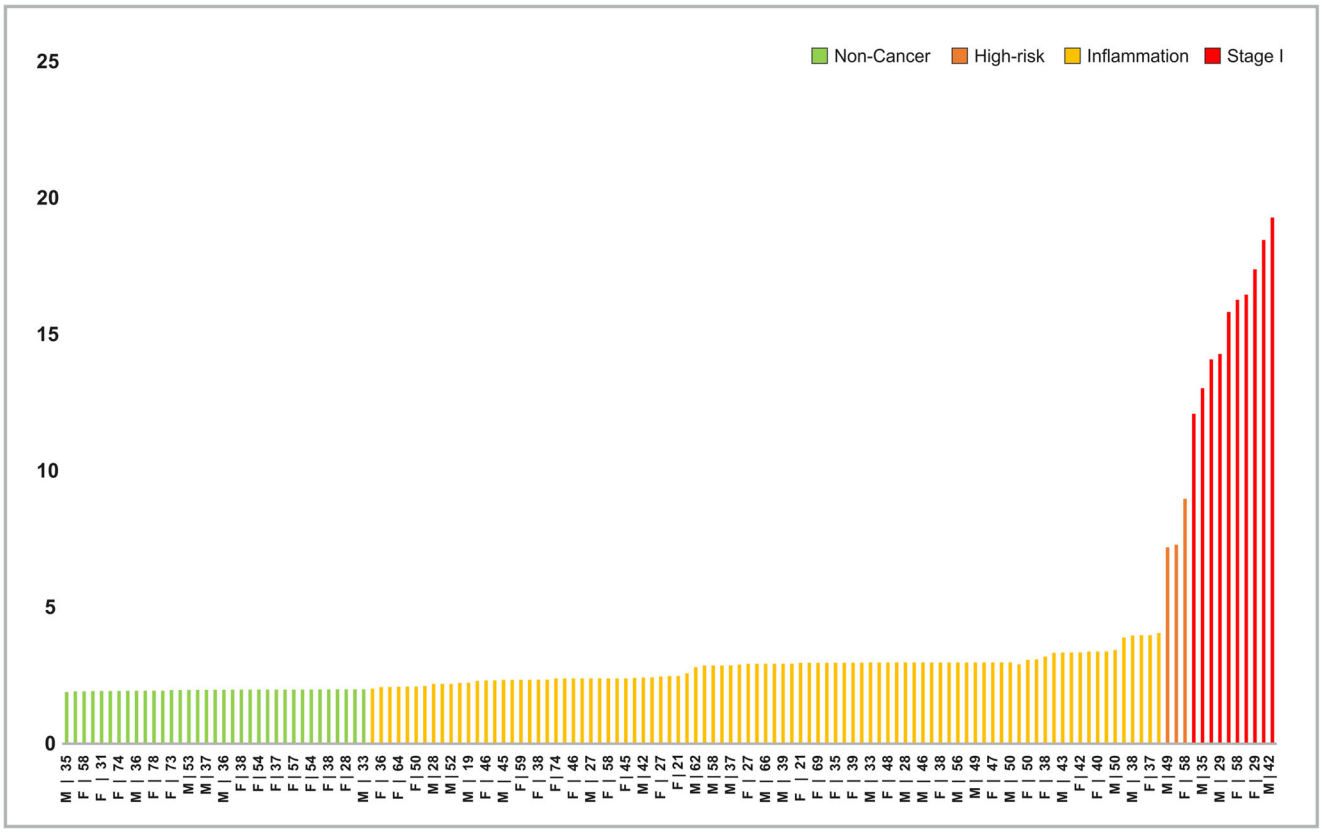

Fig. 6 Distribution of subjects aligned on the basis of their HrC values arranged in ascending order and identified as non-cancer, Inflammation, high risk and Stage I cancer

molecules that provide clues about the possible site or origin [29]. However, for cancer diagnosis, exosomes suffer from several disadvantages including ultracentrifugation methods required to isolate them might involve contamination with

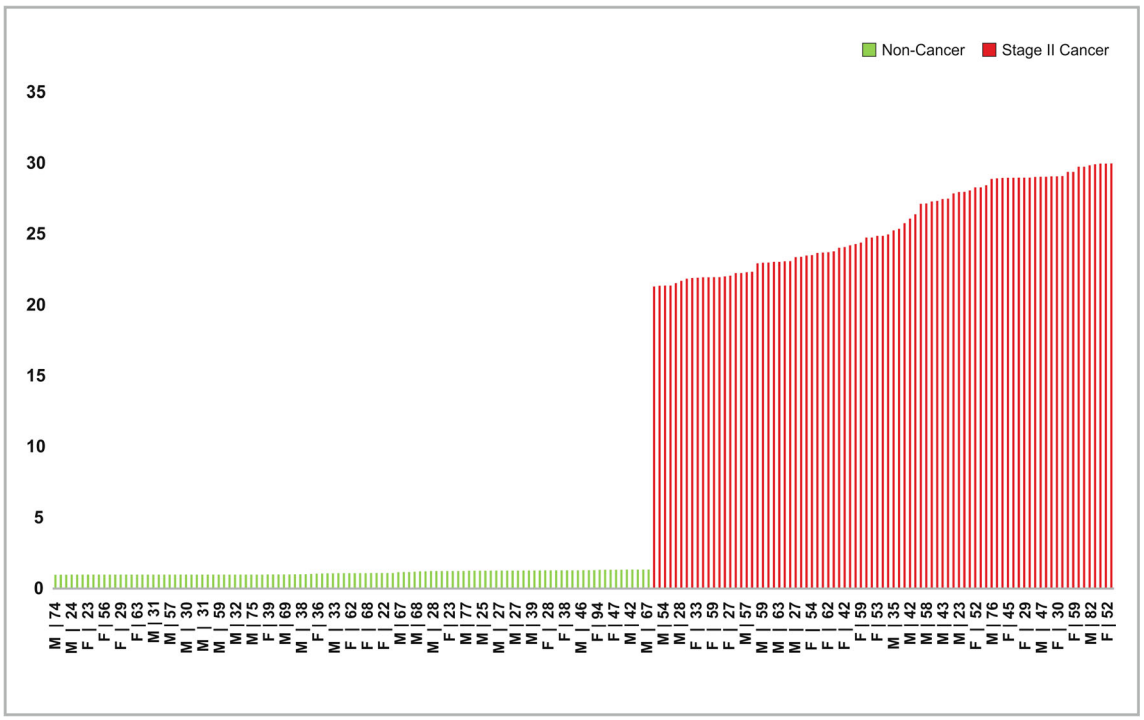

Fig. 7 Distribution of subjects aligned on the basis of their HrC values arranged in ascending order and identified as non-cancer and stage II cancer

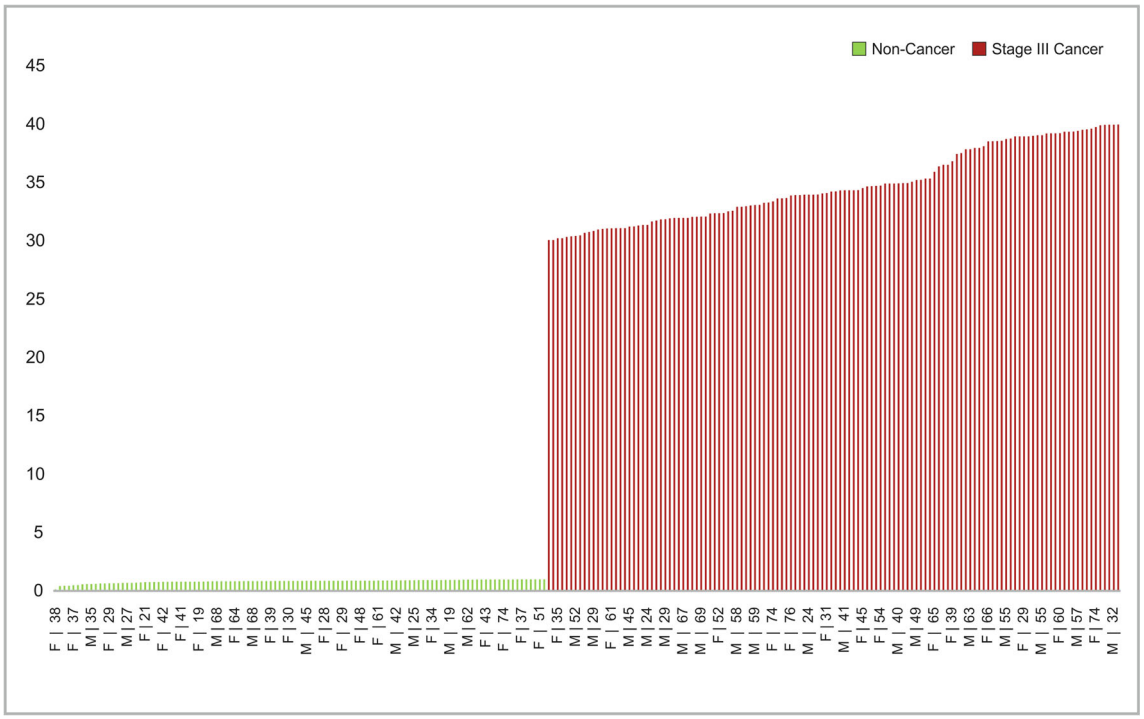

Fig. 8 Distribution of subjects aligned on the basis of their HrC values arranged in ascending order and identified as non-cancer and stage III cancer

microparticles and apoptotic bodies, thus disturbing the results; most studies involve single time point for exosomal markers as identification of tumor and it is unable to detect the different stages of cancer; this method possesses lower sensitivity and specificity compared to HrC test for presence, absence, and staging of cancer. Moreover, DNA methylation markers do not reflect accurate depiction of all cancer subtypes. Thus, it is prudent to utilize normal cells from whole blood to diagnose cancer stages and their classification using specific biomarkers.

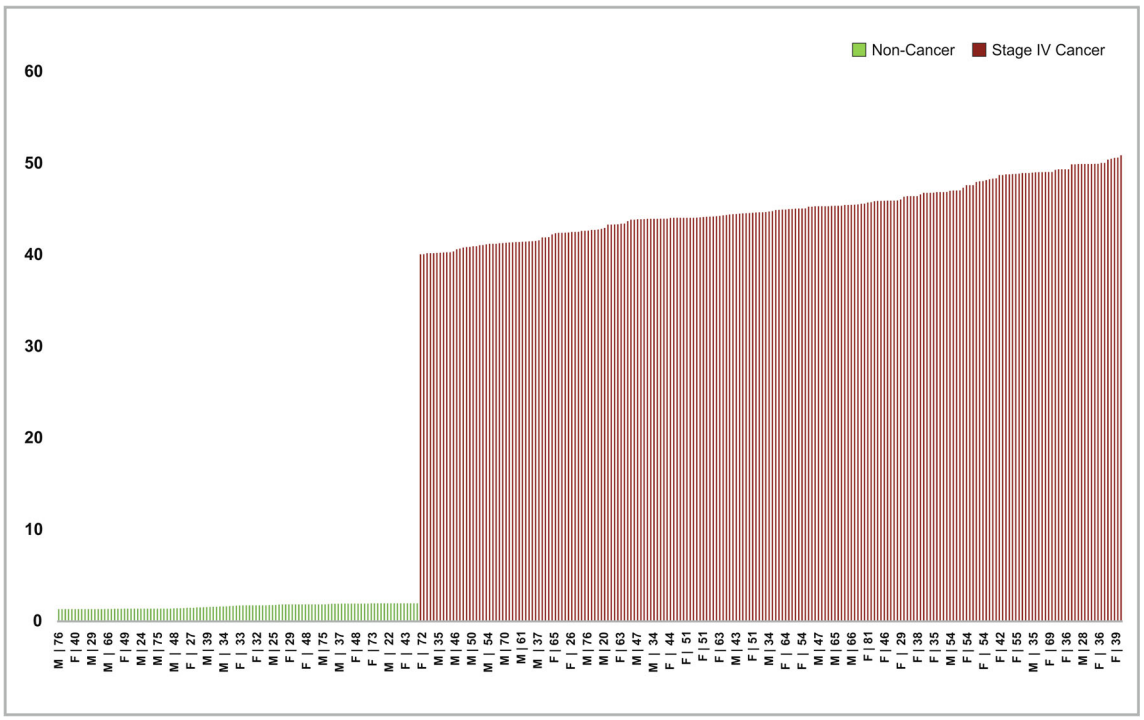

Fig. 9 Distribution of subjects aligned on the basis of their HrC values arranged in ascending order and identified as non-cancer and stage IV cancer

Evidently OCT-4 expression in circulation will be increased in all types of cancers and will need to be correlated with patient history. VSELs are expected to differentiate into progenitors which are tissue specific. Thus, the site of origin of cancer can be deduced by studying the transcriptome of enriched VSELs. Our initial NGS data (not shown here) in a few cases helped provide patient specific information. NGS studies on total cells isolated from the peripheral blood may not yield similar information. Stem cells comprise less than 1% of total cells and their transcriptome will never get deciphered while studying RNA extracted from total cells. This is the highlight of the present study. Both OCT-4A expression and NGS were carried out on an enriched population of OCT-4 expressing CSCs or transformed VSELs in the peripheral blood.

Once we understand cancer, what are CSCs, how CSCs or the 'transformed' VSELs differ from the VSELs in normal tissues, we will be able to win the war against cancer. Whether VSELs are mobilized in cancer patients recognizing growing tumor as injury with an attempt to provide vasculature and stroma as suggested by Ratajczak's group [6] or are responsible for metastasis in distant tissues are open questions that require further research. Cancer treatments at present remain very non-specific, target cancer cells rather than the CSCs and this results in recurrence. Strategies need to be developed to target the CSCs and revert them back to VSELs. For this one needs to think of stem cells along with their niche. Our study provides newer perspectives for further research. To conclude, the HrC scale reported in the present study offers an excellent non-invasive tool to better manage cancer patients.

VSELs could be just mobilized in cancer patients recognizing growing tumor as injury - with an attempt to provide vasculature and stroma.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12015-021-10167-1.

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Code Availability Not applicable.

Authors' Contributions AR, AB and RD recruited subjects and reviewed the paper. DB wrote sections of the paper and reviewed it. VKT, AV and PP reviewed the paper. SC, VT performed laboratory experiments, analysed the data and reviewed the paper. NS reviewed the paper and drafted sections. BC performed statistical analysis. AT wrote sections as well as reviewed the paper.

Data Availability We have all the data and material available with us.

Declarations

Ethics Approval Ethics approval from Ethics Committee of Maharashtra Technical Education Society at Sanjeevan Hospital, Pune, India and was registered with Clinical Trial Registry India (CTRI/2019/01/017166).

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent for Publication Patients signed informed consent regarding publishing their data.

Conflict of Interest The HrC scale was co-developed by Epigeneres Biotech. The pilot clinical study was sponsored by Epigeneres Biotech. The IP for the technology is owned by 23Ikigai.

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