Microenvironment modulated metastatic CD133+/CXCR4+/EpCAM- lung cancer initiating cells sustain tumor dissemination and correlate with poor prognosis

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ABSTRACT
Metastasis is the main reason for lung cancer related mortality but little is known about specific determinants of successful dissemination from primary tumors and metastasis initiation. Here we show that CD133+/CXCR4+ cancer initiating cells (CICs) directly isolated from patient-derived xenografts (PDX) of non-small cell lung cancer (NSCLC) are endowed with superior ability to seed and initiate metastasis at distant organs. We additionally report that CXCR4 inhibition successfully prevents the increase of cisplatin-resistant CD133+/CXCR4+ cells in residual tumors and their metastatization. Immunophenotypic analysis of lung tumor cells intravenously injected or spontaneously disseminated to murine lungs, demonstrates the survival advantage and increased colonization ability of a specific subset of CD133+/CXCR4+ with reduced expression of epithelial cell adhesion molecule (EpCAM-), that also shows the greatest in vitro invasive potential. We next prove that recovered disseminated cells from lungs of PDX bearing mice enriched for CD133+/CXCR4+/EpCAM- CICs are highly tumorigenic and metastatic. Importantly, microenvironment stimuli eliciting EMT, including signals from cancer-associated fibroblasts, are able to increase the dissemination potential of lung cancer cells through the generation of CD133+/CXCR4+/EpCAM- subset. These findings also have correlates in patient samples where disseminating CICs are enriched in metastatic lymphnodes (20-fold, $p=0.006$) and their detection in primary tumors is correlated with poor clinical outcome (DFS $p=0.03$; OS $p=0.05$). Overall these results highlight the importance of specific cellular subsets in the metastatic process, the need for in depth characterization of disseminating tumor cells and the potential of therapeutic strategies targeting both primary tumor and tumor-microenvironment interactions.
INTRODUCTION

Despite novel therapeutic strategies, survival rates for lung cancer have not greatly improved in recent years mostly due to late diagnosis and limited efficacy of available pharmacological treatments (1). Cancer initiating cells (CICs), functionally defined as the subset of cancer cells responsible for tumor generation and maintenance (2,3), could also account for tumor recurrence and metastatic seeding due to their inherent stem-like and chemoresistant properties (4). In this context, the subset of tumor cells endowed with both stemness and motility properties, possibly responsible for metastasis development, has been defined as migrating CICs (5,6).

Interestingly activation of the epithelial to mesenchymal transition (EMT) process, which is pivotal in metastatic progression, has been shown to modulate stemness properties of cancer cells indicating a possible connection between external cues and the generation of potential seeds of metastases (7-9). The subset of CICs deputed to tumor dissemination and metastasis could therefore be modulated by tumor microenvironment stimuli possibly through EMT activation, as recently observed for colon metastatic CICs (10).

Recent studies in transgenic mouse models also show that dissemination is an early event in tumor progression and is supported by tumor cells that have undergone EMT and possess CICs features (11,12).

Rising evidence also shows that chemotherapy might select for intrinsically chemoresistant CICs that could be responsible for primary tumor recurrence (13,14). Accordingly, we previously demonstrated that a distinct subset of CD133+ lung CICs co-expressing the chemokine receptor CXCR4 is spared by cisplatin treatment (15). The CXCL12/CXCR4 axis plays a crucial role in mediating tumor cells survival and the metastatic process (16); more recently it has been proven to sustain CICs,
through activation of self-renewal pathways (17) and to guide their metastatic dissemination in a human pancreatic tumor model (6).

In the present study through the analysis of a variety of lung cancer patients-derived xenografts (PDXs) (18) and clinical samples, we phenotypically characterize and functionally prove the existence of a novel subset of CICs, regulated by the microenvironment, driving lung tumor dissemination and metastasis and we identify CXCR4 signalling as a potential target for novel therapeutic strategies.

**MATERIALS AND METHODS**

**List of antibodies**

Antibodies used for FACS analysis were: anti-human PE CD133/1, FITC CD326 (EpCAM) (Miltenyi Biotech), APC CD187 (CXCR4) (BD Pharmingen), anti-mouse PerCP-eFluor 710 MHC class I, anti-human PerCP-eFluor 710 CD31 (eBioscience) and anti-human PC5 CD34, PC5 CD45 (Beckman Coulter). Anti-human FITC cytokeratin (CK3-6H5, Miltenyi Biotech) was used for immunofluorescence (IF) staining. For immunohistochemical (IHC) analyses following antibodies were used: anti-human CD133/1 (AC133, Miltenyi Biotech), CXCR4 (CXC-4000-RM, Biotrend), Keratin, Pan Ab-1 (Clone AE1/AE3, Dako), CD34 (clone QbndN/10, Neomarkers) and low molecular weight cytokeratins (clone AE1/AE3/PCK26, Ventana Medical Systems). Detailed protocols for flow cytometry, IF and IHC staining are Supplemental Materials and Methods.
**Tissue dissociation and culture**

Clinical specimens were obtained from a consecutive series of 97 consenting patients (17 also had matching lymphnodes available). The protocol was approved by the Internal Review and the Ethics Boards of the Fondazione IRCCS Istituto Nazionale Tumori of Milan.

The protocol for primary tumors, lymph nodes, PDXs and murine lungs tissue dissociation was already described in (15) and detailed in Supplemental Materials and Methods.

Culture method to obtain cancer tissue originated spheroids (CTOS) and murine lung tissue spheroids was adapted from Kondo J et al. (19). Briefly, enzymatically digested tumor tissue or murine lungs were subsequently filtered through 100-μm and 40-μm cell strainers (BD Bioscience). Tissue organoids retained in the 40-μm strainers were washed in 30ml of DMEM/F12 medium (Lonza) and centrifuged at 100g for 5min. Organoids were plated in stem cell medium SCM (described in (15)) in a 60mm dishes. For culture expansion, spheroids were mildly split in smaller pieces, avoiding single cells dissociation, by incubation with collagenase IV (5mg/ml) (Sigma) at 37°C for 5min.

**Cell lines and treatments**

All tumor cell lines were cultures in RPMI with 10% fetal bovine serum (FBS) at 37°C under 5% CO2. LT73 primary cell line was derived from a surgical specimen of a male patient with lung adenocarcinoma. H460 cell line (lung large-cell carcinoma) was purchased from American Type Culture Collection (ATCC) and authenticated by DNA short tandem repeat (STR) profiling.

See Supplemental Materials and Methods for more details.
To induce EMT, cell cultures were treated with TGFβ (5ng/ml Peprotech) for 5 up to 15 days.

CTCE-9908 inhibitor and corresponding scramble control peptide were kindly provided by British Canadian BioSciences Corp. and used for in vitro cells cultures treatment at 10μg/ml for 30min at 37°C in the appropriate growing medium (RPMI-1640 plus 1% FBS or in stem cells medium), before FACS analysis or in in vitro invasion assay, detailed in Supplemental Materials and Methods.

Animal studies

In vivo experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale dei Tumori, according to institutional guidelines.

Patient derived xenografts were established from primary lung cancers from patients undergoing surgical resection, who gave their informed consent and expanded as previously described (18).

For in vivo combination therapy, cisplatin (Teva Pharma) was administered i.v. at 5mg/kg every 7 day for 3 weeks, starting when tumors were palpable (>=50mm³). CTCE-9908 was administered i.p. (50mg/kg) once every 5 days post cisplatin administration for 3 weeks.

Human-in-mice model of bone metastasis was established as previously reported (20). Bone metastasis assay was performed by injecting subcutaneously close to human bone implant 1 x10⁵ cells sorted from PDX models for CD133 and CXCR4 or by intracardiac (i.c.) route, injecting in the left ventricle 1 x10⁴ sorted cells in 100 μL physiological solution.
Additional information on PDX models and in vivo experiments was detailed in Supplemental Material and Methods.

**Integrated strategies to detect DTCs in murine lung**

In order to set up the most robust strategy to detect disseminating tumor cells (DTCs) in murine lungs, i.v injection of scalar doses of H460 cells was performed in SCID mice (n=2 mice for each dose) and lungs were analyzed 2h post injection by FACS, Real Time PCR and IHC.

For FACS analysis, injected tumor cells were identified using a negative gating strategy to exclude 7-AAD<sup>+</sup> dead cells and mouse H2K<sup>+</sup> cells that was able to specifically detect as few as 10<sup>3</sup> single tumor cells in murine lungs (Supplementary Fig. S1A. and Supplemental Materials and Methods).

For detection of tumor cells in murine lungs by Real Time PCR, analysis of human-specific β2 microglobulin (B2M) housekeeping gene expression was performed, using murine B2m for cDNA normalization of samples. This analysis reliably detected to a lower limit of 10<sup>3</sup> tumor cells in murine lung. (Supplementary Fig. S1B and Supplemental Materials and Methods).

IHC analysis on formalin-fixed paraffin embedded (FFPE) lung tissue for pan-cytokeratins (CKs) expression was able to robustly identify single cells when injected at 10<sup>4</sup>. To detect lower number of single cells at least 5 consecutively sections of FFPE lung tissue, cut at 1.5μm thickness, must be analyzed (Supplementary Fig. S1C).
RESULTS

The subset of CD133+CXCR4+ lung CICs is endowed with increased seeding and metastatic potential

To verify the possible existence of a metastatic subset in lung cancer initiating cells (CICs), we analyzed by FACS 78 primary human non small cell lung cancer (NSCLC) for CD133 and CXCR4 expression: double positive cells accounted for 0.3% of total tumor cells (median value, range 0%-8%) and represented 20% of CD133+ cells (median value, range 0%-100%). Interestingly, when primary tumors were compared to matched lymph-node metastasis (n=17), the frequency of CD133+ cells co-expressing CXCR4 was significantly higher in metastases (3-fold enrichment, p=0.01) (Fig. 1A), suggesting a relevant role in the early steps of tumor dissemination.

To dissect the metastatic potential of different subpopulations of lung CICs, CD133+CXCR4+ and CD133+CXCR4- cells sorted from different PDXs (n=5), from a primary cell line freshly established from a lung adenocarcinoma sample (Lung Tumor 73-LT73) and from H460 long-term established cell line were injected in the tail vain of SCID mice. CD133–CXCR4– non-CICs were used as control. To detect and monitor during time tumor cells in murine lungs we set up and validated a method based on flow cytometry analysis, not relying on specific human antigens expression and supported by RT-qPCR and IHC analysis (Supplementary Fig. S1 A-C). Three months post injection, rare tumor cells were detectable in murine lungs and were consistently higher in the groups injected with CD133+CXCR4+ (2-fold increase +/+ vs. +/-; 5-fold increase +/+ vs. -/-, p=0.001) (Fig. 1B). After an extended period of time (six months) CD133+CXCR4+ cells, sorted from one PDX model (LT111) and LT73 and H460 cell lines, showed their full metastatic potential and
greatest ability to colonize murine lungs compared to other injected groups as revealed by both FACS analysis (4.5 fold-increase, \( p=0.0003 \) +/+ vs +/-; 6.4 fold-increase, \( p=0.0006 \) +/+ vs -/-) (Fig. 1B) and histological analysis (Fig. 1C): software-assisted quantification of macroscopic areas of tumor invasion clearly demonstrated the higher degree of infiltration of lung parenchyma of double-positive cells compared to moderate and slight degree of infiltration of single-positive and double-negative cells (\( p=0.0003 \) +/+ vs. +/-; \( p=0.0004 \) +/+ vs. -/-) (Fig. 1D). A relevant inflammatory infiltrate, mainly consisting of polymorphonuclear leukocytes, was also observed associated with tumor foci (Fig. 1C) possibly explaining the long latency periods before metastatic cells proliferation. The phenotype of injected double positive cells remained unchanged during early colonization steps, implying that their survival advantage enables them to support metastasis initiation (Fig. 1E).

To confirm the high metastatic properties of CD133+CXCR4+ cells and their selective advantage in seeding different organs, we exploited an innovative model of humanized mice implanted with human bone (20,21), the second more frequent metastatic site of NSCLC. The implanted human bone, detected in the mouse flank by X-rays examination, was viable as indicated by the presence of bone marrow cells, mineralized areas, stromal cells and neo-vascularization (Fig. 2A). Unsorted tumor cells and CD133+CXCR4+, CD133+CXCR4− and CD133−CXCR4− subsets were isolated from two PDXs (LT59 and LT111) and injected both subcutaneously (s.c.), and intracardially (i.c.) in mice bearing bone, to model different aspects of bone seeding. CD133+CXCR4+ cells were able to colonize human bone generating metastatic lesion after both s.c. and i.c. injection with high efficiency (65%, 9/14 mice), conversely CD133+CXCR4− and CD133−CXCR4− cells did not (Fig. 2B and Supplementary Table S1). Enhanced bone remodeling, revealed by large areas of
new bone apposition and an increased osteoid thickness due to metastasis formation, was evident in mice injected with CD133⁺CXCR4⁺ cells compared to all other groups (p<0.05) (Fig. 2C and D). Notably, CD133⁺CXCR4⁺ cells injected both s.c. and i.c. in mice carrying human bone implants also showed a significantly higher ability to disseminate and initiate lung metastasis, with extensive replacement of normal lung parenchyma with cancer cells (Fig. 2E), compared to CD133⁺CXCR4⁻, CD133⁻CXCR4⁻ cells or unsorted cells (CD133⁺CXCR4⁺ vs. others groups P=0.007) (Supplementary Table S1).

Altogether these results prove the high dissemination and colonization potential of CD133⁺CXCR4⁺ cells and their ability to support metastasis development at distant organs.

**Inhibition of the CXCR4 pathway prevents metastatic spread of chemoresistant CD133⁺CXCR4⁺ cells**

In an attempt to block the migration of the CD133⁺CXCR4⁺ metastatic subset, we tested the CXCR4 inhibitor CTCE-9908, a small peptide analog of SDF-1, previously shown to be effective in preventing metastatic dissemination in different cancer models (22-25).

*In vitro* CTCE-9908 treatment could block the invasion of PDX-derived tumor cells (n=4 PDX models) induced by both SDF-1 and FBS (Fig. 3A). Interestingly we also verified that the invading fraction of PDXs cells attracted with a non-specific signal (FBS) was 10-fold enriched in CD133⁺CXCR4⁺ cells compared to total population and that CTCE-9908 inhibitor was able to specifically block their invasion (4-fold change) (Fig. 3B), thus pointing at CXCR4 axis as a pivotal mediator of lung CICs invasion.
To test the efficacy of CXCR4 inhibition in a clinically relevant therapeutic setting, we treated *in vivo* metastatic H460 xenografts with CTCE-9908 in combination with standard chemotherapy. We previously reported that cisplatin treatment, although effective in reducing tumor size, induces enrichment in the residual tumor of chemoresistant CICs (15). Combination treatment with CTCE-9908 had a similar efficacy in inhibiting subcutaneous tumor growth as cisplatin (Supplementary Fig. S2A), but it was able to prevent the relative increase of CD133+CXCR4+ cells and concomitant up-regulation of stemness genes induced by chemotherapy alone (Fig. 3C and D).

Of note, FACS analysis of H460 lung metastasis in control mice demonstrated a 10-fold enrichment for CD133+CXCR4+ cells compared to matched s.c. xenograft (p<0.05), confirming their pivotal role also in the spontaneous metastasis development (Supplementary Fig S2B).

Cisplatin treatment primed the dissemination of CD133+CXCR4+ subset (2-fold change) inducing a boost in metastasis formation (1.8-fold change) compared to control group (Fig. 3E and F). Combination therapy with CXCR4 inhibitor was able to counteract cisplatin effect, drastically reducing metastatic formation (2-fold decrease) and concomitantly decreasing the subsets of CD133+ and CD133+CXCR4+ cells in lung metastasis compared to cisplatin-treated group (2.6 and 2.3-fold change respectively) (Fig. 3E and F and Supplementary Fig. S2C).

Next, we proved also in highly disseminating PDX model (LT111) that *in vivo* combination treatment with CTCE-9908 had a modest effect on tumor growth inhibition (Supplementary Fig. S2D), but was able to prevent tumor spread associated with cisplatin treatment (1.5-fold change) and to efficiently reduce the
fraction of disseminated CD133⁺CXCR4⁺ CICs to murine lungs (4.7-fold change) (Fig. 3G and Supplementary Fig. S2E).

We additionally verified in cell lines that CXCR4 is also frequently expressed (mean 70%, range 50-90%) in another subset of CICs previously identified as chemoresistant (CD133⁺ABCG2⁺) (15), and that CXCR4 inhibition could also prevent enrichment of CD133⁺CXCR4⁺ABCG2⁺ induced by chemotherapy (data not shown).

Our results suggest that combination treatment with CXCR4 inhibitor could specifically impair dissemination of chemoresistant and metastatic CD133⁺CXCR4⁺ cells.

**CD133⁺CXCR4⁺ and EpCAM⁻ CICs have survival advantage at ectopic sites in early steps of colonization**

To examine the role of CD133⁺CXCR4⁺ cells in different steps of the metastatic cascade, we injected i.v. 1x10⁶ unsorted lung tumor cells endowed with different metastatic behavior (LT111-PDX cells=weakly metastatic; LT73 cell line=moderately metastatic; H460 cell line=highly metastatic) and we monitored in time course experiment which tumor cell subsets were preferentially selected to drive specific steps of metastasis development.

Flow cytometry analysis of murine lung 2h post injection confirmed the presence of tumor cells that maintained the same phenotype of original tumor cells (Supplementary Fig. S3A). After 1 week the number of tumor cells drastically decreased (20-fold) with a concomitant enrichment for CD133⁺CXCR4⁺ subset in the surviving tumor fraction (Fig. 4A). We also detected in LT73 and LT111 surviving cells a significant increase for a fraction of CD133⁺ CICs negative for the expression of epithelial antigen EpCAM compared to parental injected tumor cells (18-fold
increase), whereas parental H460 cell line does not express at all EpCAM marker (Fig. 4A). Surviving LT111 cells remained in murine lung, without proliferating, for up to 3 months and stably showed enrichment for CD133\(^+\)CXCR4\(^+\) and CD133\(^+\)EpCAM\(^-\) subsets (Fig. 4A). Conversely, during metastatic growth of LT73 and H460 cells, both subsets of CD133\(^+\)CXCR4\(^+\) and CD133\(^+\)EpCAM\(^-\) cells, diminished and reverted to the original percentage of the parental cell line when metastases reached macroscopic size (Fig. 4A and Supplementary Fig. S3B). Expression of stemness genes followed the same trend of CD133\(^+\)CXCR4\(^+\) cells in the different phases of metastatic development (Fig. 4B).

In summary these findings confirm the primary role of CD133\(^+\)CXCR4\(^+\) CICs in metastasis process, due to a superior survival advantage and colonization ability, and reveal the existence of CD133\(^+\)EpCAM\(^-\) subset strongly enriched in early steps of colonization.

The subset of CD133\(^+\)CXCR4\(^+\)EpCAM\(^-\) CICs sustains lung cancer dissemination and is endowed with high tumorigenic and metastatic potential

To further investigate the involvement of CD133\(^+\)CXCR4\(^+\) and EpCAM\(^-\) subset in pre-clinical model of spontaneous tumor dissemination, we analyzed lungs of PDX-bearing SCID mice.

While generally no histological metastases were evident, FACS analysis was able to detect a subset of disseminated tumor cells (DTCs) in 9/11 PDX models (n= 6 adenocarcinoma, ADC; 1 large cell carcinoma, LCC; 2 squamous cell carcinoma models, SCC), in a percentage (range 0.001%-0.01%) consistent with that estimated from human B2M expression by RT-qPCR (data not shown).
Interestingly the fraction of lung DTCs was already detectable at early stage of subcutaneous tumor development and remained stable during tumor growth (Supplementary Fig. S4A). Notably, immunophenotyping of lung DTCs revealed a constant increase for the fraction of CD133⁺CXCR4⁺ CICs (p=0.04) and in particular a predominant enrichment for the subset of CD133⁺EpCAM⁻ cells (p=0.007) compared to parental subcutaneous tumors (Supplementary Fig. S4A and B), confirming evidences already reported in i.v. time course experiments.

Moreover, in vitro invasion assay on dissociated PDX cells (n=5) proved that CD133⁺CXCR4⁺EpCAM⁻ cells represented the CICs subset more significantly enriched in the invading fraction compared to total population (90-fold, p=0.01) (Fig. 5A).

To functionally characterize the subset of lung DTCs enriched in disseminating CICs, we adapted an in vitro culture method useful to enrich for tumor cells (19): from 6 different PDXs (4 ADC, 1 SCC, 1 LCC) we generated cultures of cancer tissue originated spheroids (CTOS) and corresponding lung tissue spheroids, highly enriched in DTCs compared to freshly dissociated lung tissue (range 70-145 fold increase) as also confirmed by RT-qPCR analysis for hB2M expression and by staining for human cytokeratins (Fig. 5B and Supplementary Fig. S4C). Real time analysis of lung spheroids cultures showed a strong up-regulation for stemness genes and for a panel of EMT-related genes in DTCs compared to matching PDX-CTOS, subtending an enrichment for the subset of CD133⁺ stem-like cells endowed with mesenchymal features (i.e. EpCAM neg) (Fig. 5C).

To functionally test tumorigenic properties of DTCs, we subcutaneously injected in NOD-SCID mice 1x10⁵ lung spheroids dissociated cells, containing approximately 200 DTCs and 1x10⁵ PDX-CTOS dissociated cells generated from LT59 PDX. DTCs
were able to originate tumors that closely resembled histology and phenotype of original PDX (Fig. 5D), but contained a larger amount of CD133\(^+\)CXCR4\(^+\)EpCAM\(^-\) cells compared to parental xenograft (Fig. 5E). Moreover, cells from DTCs-tumors maintained a slightly increased expression of EMT-related genes compared to CTOS-tumors and exclusively possessed the ability to proliferate \textit{in vitro} as spheres in anchorage independent condition (Supplementary Fig. S4D and E), features associated with a mesenchymal and invasive phenotype.

Remarkably, \textit{in vivo} s.c. injection of cells from DTCs-tumors demonstrated an enhanced ability to generate secondary tumors (Fig. 5F) and higher lung dissemination ability compared to CTOS-tumor (disseminated tumor cells: 0.6\(\pm\)0.1 vs. 0.09\(\pm\)0.03) (Fig. 5G).

Finally intravenous injection of LT59 lung spheroids dissociated cells (~200 DTCs) in NOD-SCID mice generated lung metastases, which again showed an increased fraction CD133\(^+\)CXCR4\(^+\)EpCAM\(^-\) cells, whereas up to \(1 \times 10^5\) LT59 cells and LT59-CTOS cells failed to initiate metastasis (Fig. 5H).

Our evidences prove the existence of invasive and highly tumorigenic CD133\(^+\)CXCR4\(^+\)EpCAM\(^-\) subset endowed that specifically drives lung tumor dissemination.

**Microenvironment stimuli modulate the subset of CD133\(^+\)CXCR4\(^+\)EpCAM\(^-\) lung disseminating CICs**

The mesenchymal properties displayed by CD133\(^+\)CXCR4\(^+\)EpCAM\(^-\) disseminating CICs, lead us to investigate the possible involvement of EMT activation in their generation/modulation, since the link between EMT and acquisition of migratory and stemness features is well recognized (26).
Induction of EMT through TGFβ treatment of LT73 tumor cell line (LT73-EMT), evaluated as morphological changes, modulation of EMT-related genes and increased invasive potential (Supplementary Fig. S5A-C), caused a ten-fold increase in the fraction of CD133+ CICs (p=0.0001) with a concomitant up-regulation of stemness genes expression (Fig. 6A and Supplementary Fig. S5D). We also verified that LT73-EMT cells showed an expression pattern for EMT and stemness-related genes more similar to mesenchymal stem cells (MSC) then to differentiated adult mesenchymal cells (i.e. fibroblasts derived from primary lung tumors) (Fig. 6B).

Increase of CD133+ cells in LT73-EMT cells was functionally proved by enhanced in vivo tumorigenic potential and ensued tumors stably maintained an augmented content of CD133+ cells compared to LT73 parental tumors (Supplementary Fig. S5E and F).

Examination of CD133+CICs subsets in LT73-EMT cells, demonstrated a 3.8-fold increase (p=0.04) for metastatic CD133+CXCR4+ cells, that was mainly driven by the expansion of CD133+CXCR4+EpCAM− subpopulation (13.5-fold increase, p=0.02) (Fig. 6C).

We confirmed also in PDXs and primary CTOS cultures (n=5) that EMT induced by TGFβ was able to enrich for CD133+CXCR4+EpCAM− disseminating CICs (15-fold change, p=0.05) (Fig. 6D and Supplementary Fig. S5G), associated with an increased in vitro invasiveness and up-regulation of EMT and stemness genes (Supplementary Fig. S5H and I).

To evaluate whether the CICs enrichment was due to an expansion of the pre-existing CD133+ pool or to de-novo generation of positive cells, we deplete CD133+ cells by FACS sorting from LT73 cell line, creating LT73 CD133neg line that stably maintained a CD133 negative phenotype during in vitro culturing (Fig. 6E).
TGFβ treatment of LT73 CD133neg cell line induced the generation of a fraction of CD133+ cells (mean 0.14% ± SD 0.05%), similar to that observed in LT73 parental cell line (mean 0.09% ± SD 0.02%), that however exhibited a lower expression of EpCAM compared to endogenous CD133+ cells (Fig. 6E). Upon TGFβ withdrawal, LT73-CD133neg cells slowly reverted to an epithelial phenotype whereas the fraction of de novo generated mesenchymal CD133+ cells persisted, indicating a stable reprogramming (Fig. 6E and Supplementary Fig. S5L and M). Consistently, tumors derived from s.c. injection of LT73 CD133neg-EMT appeared with a shorter latency period then LT73 CD133neg cells and maintained a similar fraction of de novo generated CD133+ cells (Supplementary Fig. S5N and O).

To verify whether physiologically relevant tumor microenvironment stimuli could show similar proficiency in generating lung CICs, LT73-CD133neg cells were co-cultured with conditioned medium (CM) from cancer associated fibroblasts (CAF) isolated from primary human lung tumors. A variable but constantly detectable fraction of de novo CD133+ cells was observed after exposure to different CMs, mirrored by a parallel modulation of EMT genes (Fig. 6F and G) indicating that also stimuli from microenvironment are able to endow tumor cells with mesenchymal traits associated with CICs generation.

Since stromal SFD-1 has been proven to induce EMT activation in different tumor types (27,28), we explored the role of SDF-1 as a possible mediator of CAFs effects on tumor cells. In line with reported evidences, we confirmed that in vitro treatment of LT73 cell line with SDF-1 resulted in up-regulation of EMT genes (Supplementary Fig. S5P). Treatment of LT73 CD133neg cells with CMs and CXCR4 inhibitor CTCE-9908 demonstrated a partial prevention of EMT genes modulation compared to CMs
alone, thus confirming that SDF-1 represents one of the CAFs-secreted factors that mediate EMT-activation in tumor cells (Fig. 6G).

CM-generated CD133⁺ CICs were functionally associated with an enhanced in vivo tumorigenicity compared to untreated CD133neg cells (Fig. 6H) and interestingly the ensuing xenografts displayed a CD133 fraction reflecting the proficiency of CAF-CMs to create CICs in vitro (Fig. 6I). Notably, the relative percentages of de novo generated CD133⁺CXCR4⁺EpCAM⁻ cells detected in CM-treated LT73CD133neg tumors correlated with their disseminating potential to murine lungs (Fig. 6I and L).

Our data demonstrate a dynamic phenotype of lung CICs and show that stimuli from tumor microenvironment primarily generate the subset of CD133⁺CXCR4⁺EpCAM⁻ CICs functionally associated with tumor dissemination.

**Disseminating CICs can be detected in lymphnodes of lung cancer patients and correlate with poor clinical outcome**

Comparison of lymph node metastases and matched synchronous primary NSCLC tumors (n=17) revealed a 20-fold enrichment in the fraction of CD133⁺CXCR4⁺EpCAM⁻ cells in metastases (p=0.006) (Fig. 7A), confirming in a clinical setting our pre-clinical data for the existence of CD133⁺CXCR4⁺EpCAM⁻ CICs related to tumor dissemination and metastasis initiation.

To evaluate the potential clinical relevance of disseminating CICs we analyzed by FACS 78 surgically resected primary NSCLC for expression of CD133, CXCR4 and EpCAM. As we previously reported, the overall frequency of CD133⁺ cells was generally low with a median value of 1.3% (inter-tertile range: 0.7-2%). A subset of CD133⁺CXCR4⁺EpCAM⁻ cells was detected in 50% of cases, independently from the total amount of EpCAM⁺ tumor cells. The CD133⁺CXCR4⁺EpCAM⁻ fraction
accounted for 12.5% of total CD133+ cells (range 0.4%-100%). There was no correlation among the total amount of CD133+ cells and clinical variables including age, sex, histological subtype, grade or stage (Supplementary Table S2). The frequency of migrating CICs (CD133+CXCR4+EpCAM−) within the CD133 pool was more frequent in squamous cell carcinomas (p=0.017). Interestingly both CD133+ and CD133+CXCR4+EpCAM− content was associated with presence of chronic obstructive pulmonary disease (p=0.025 and p=0.011 respectively) indicating a possible role of the lung microenvironment in modulating stemness and disseminating properties. More importantly while the total amount of CD133+ cells did not correlate with clinical outcome, the presence of the CD133+CXCR4+EpCAM− subset was negatively correlated with both disease free survival (DFS, p=0.033) and overall survival (OS, p=0.055) (Fig. 7B). The association of disseminating CICs with DFS remained significant also in subclass analyses when only non-squamous tumors were analyzed (p=0.038) and in a multivariate model even after adjustment for stage (p=0.03, HR:2.25, 95% CI: 1.05-4.82). Particularly in tumors with low content of CD133+ cells (≤2%) presence of CD133+CXCR4+EpCAM− metastatic CICs was associated in multivariate analysis with both shorter DFS (p=0.03, HR:3.34, 95%CI: 1.09-10.18) and OS (p=0.05, HR:3.10, 95%CI: 1.00-9.59), thus representing a risk factor for tumor relapse and poor prognosis in NSCLC patients.

**DISCUSSION**

Although metastasis has generally been considered a multistep process requiring acquisition of specific genetic alterations providing tumor cells with crucial advantages (29), plasticity is gaining attention as a fundamental trait for metastasis–
prone tumors and the identification of definite phenotypes linked to this process appears crucial for its understanding (30).

Here we demonstrate that the subset of CD133⁺CXCR4⁺ lung CICs drives tumor cells seeding and metastasis initiation in lung cancer. The high metastatic potential of CD133⁺CXCR4⁺ cells and their enhanced ability to seed and colonize different organs (i.e. lung and bone) is substantiated in experimental lung metastasis as well as in an humanized murine model of bone metastasis: interestingly in such experimental model the presence of a “human pre-metastatic niche” represented by implanted bone is conductive for generation of overt metastases in a considerable shorter time than in experimental metastasis assay, stressing the importance of proficient niche to support and dictate tumor development and the challenge of recapitulating the human microenvironment in mouse (31-33).

Next, we prove for the first time that CXCR4 targeting is able to counteract the chemotherapy-induced metastatic spread of chemoresistant fractions of CD133⁺CXCR4⁺ and possibly also of CD133⁺ABCG2⁺ CICs (15), that mostly express CXCR4 receptors, pointing at combination therapy with CXCR4 inhibitor as an attractive novel strategy to improve neo-adjuvant and adjuvant therapy for NSCLC.

Time course analysis of phenotypic changes in i.v. injected tumor cells at different steps of metastatic cascade revealed in the first phase of colonization an enrichment for a subset of lung CD133⁺CXCR4⁺ negative for EpCAM; similar enrichment was observed in spontaneously early-disseminated tumor cells detected in PDXs pre-clinical model, data line with evidence in different tumor types for an early tumor metastatic spread guided by cells endowed with mesenchymal and stem like features (11,12,34). Beside the phenotypic characterization, we functionally prove
that lung DTCs, surviving in ectopic murine soil without metastatic outgrowth, are
indeed highly tumorigenic and metastatic when injected subcutaneously with
Matrigel or i.v. in NOD-SCID mice, proving the metastasis initiation potential of the
specific subset of CD133\(^+\)CXCR4\(^+\)EpCAM\(^-\) cells, as well as highlighting once more
the need of a favourable metastatic niche to dictate tumor growth (32,33,35).

Remarkably, tumors generated from DTCs, as well as patient’s lymphnodes
metastases, are enriched in CD133\(^+\)CXCR4\(^+\)EpCAM\(^-\) CICs subset, demonstrating in
a clinical setting the relevance of this subpopulation in metastasis formation and
strengthening the value of PDX models tumor dissemination and metastasis initiation
studies.

The mesenchymal features of disseminating tumor cells as well as the well-known
link between EMT activation and acquisition of invasiveness and stem-like features,
prompted us to investigate the possible involvement of EMT in their generation
(7,36,37). Our data indeed confirmed that EMT triggering generates CD133\(^+\) cells
and more interestingly we demonstrated for the first time that stimuli from tumor
microenvironment, specifically those from cancer-associated fibroblasts (CAF), could
dictate de novo creation of the CD133\(^+\)CXCR4\(^+\)EpCAM CICs subset directly linked to
EMT induction and acquisition of \textit{in vitro} and \textit{in vivo} increased dissemination. Similar
data proving the capability of microenvironmental stimuli to convert progenitor cells
into cancer stem cells with metastatic properties are reported in colon cancer (10).
Moreover our finding showing that CAF-secreted SDF-1 promotes EMT activation
and likely the acquisition of stemness features in lung tumor cells, in line with
evidence reported in colorectal cancer (27), strengthen the potential therapeutic use
of CXCR4 inhibitor to both prevent dissemination of metastatic CICs and their
generation through the impairment of tumor-stroma cross-talk.
The clinical relevance of our findings were demonstrated by the fact that the frequency of CD133$^+$CXCR4$^+$EpCAM$^-$ within the CICs pool in primary tumors represents an independent indicator of poor prognosis in surgically resected NSCLC patients, suggesting that dissection of the biological complexity of primary tumors will be crucial to improve clinical management of lung cancer.

The prospective to identify the fraction of metastatic CD133$^+$CXCR4$^+$ CICs negative for EpCAM expression in circulating tumor cells from lung cancer patients, holds therefore great interest and implications from both a prognostic and therapeutic point of view, as recently highlighted in breast cancer patients where isolated EpCAM$^{neg}$-CTCs showed high invasiveness and ability to generate brain and lung metastases (38-40).

In conclusion, we provide evidence for the existence of novel human lung disseminating CICs regulated by microenvironmental signals able to complete all the steps of the metastatic cascade and related to clinical recurrence. We also propose CXCR4 targeting as a novel therapeutic option in neo-adjuvant and adjuvant setting, to counteract the dissemination of chemoresistant and metastatic lung CICs.

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Reference List


Figure Legends

Figure 1. CD133\(^{+}\)CXCR4\(^{+}\) cells have an enhanced metastatic potential. (A) Percentages of CD133\(^{+}\)CXCR4\(^{+}\) cells in paired primary tumors and lymph node metastasis (n=17). \(p=0.01\) (B) Percentage of human tumor cells detected by flow cytometry in murine lungs 3 months and 6 months after i.v. injection of CD133\(^{+}\)CXCR4\(^{+}\), CD133\(^{+}\)CXCR4\(^{-}\), CD133\(^{-}\)CXCR4\(^{-}\) cells. n=13 mice per group at 3-months and n=7 mice per group at 6-months. ***=p≤0.001. (C) Representative imagines of H&E histological sections of murine lungs scanned with Aperio\(^{\text{®}}\) ePathology system, six months after injection of CD133\(^{+}\)CXCR4\(^{+}\), CD133\(^{+}\)CXCR4\(^{-}\) and CD133\(^{-}\)CXCR4\(^{-}\) cells sorted from LT111-PDX, LT73 and H460. Areas of tumor invasion for each model are shown on the right at higher magnification (40X); yellow arrows indicate representative infiltrating metastatic cells and green arrows polymorphonuclear leukocytes associated with tumor foci. (D) Invasion of murine lungs was calculated by scanning 4 different layers of each histological section in (C) and quantifying the percentage of invaded areas for each single lung with ImageScope Software. Bars are the mean percentage of invaded area ± SEM. *=p≤0.05, **=p≤0.01. (E) Representative FACS analysis of LT111 CD133\(^{+}\)CXCR4\(^{+}\) cells-generated metastasis, six months post injection: tumor cells are identified as mouse H2K\(^{\text{neg}}\) (left dot plot) and analyzed for CD133 and CXCR4 expression (right dot plot). IHC staining confirming CD133 and CXCR4 positivity is shown on the right.

Figure 2. CD133\(^{+}\)CXCR4\(^{+}\) cells efficiently initiate bone metastasis. (A) On the left, X-ray imaging of mouse implanted with human bone fragment in the flank. On the right, H&E on human implanted bone showing viable osteocytes, stromal cells, human
vessels (top panel). IHC staining for human CD34+ (lower panel) confirmed the
presence of human vessels (40Xmagnification). (B) IHC staining for low molecular
cytokeratins of bone metastasis derived from CD133+CXCR4+ cells (magnification
10X) and inset at higher magnification (60x). (C) Trichrome staining of human implanted bone in mice injected with CD133+CXCR4- (on the top) CD133+CXCR4+ (on the bottom) showing the presence of new collagen fibers (stained in blue). (D) Mean values ± SD of the osteoid thickness of human bone in CD133+CXCR4+ cells-injected mice compared to all other groups (n= 4). *=p≤0.05. (E) H&E staining of lung of mouse bearing human bone after s.c. injection of CD133+CXCR4+ cells showing massive lung metastatization (10X magnification) (on the left) and inset of tumor cells at 60X magnification (on the right).

**Figure 3.** CXCR4 inhibition prevents metastatic dissemination of chemoresistant CD133+ CXCR4+ cells. (A) Invasion assay performed on PDX cells treated with CTCE-9908 or scramble peptide and chemo attracted with SDF-1 (on the left) or FBS (on the right). After 72h, invaded cells were counted in 4 random fields of the inserts; aata are the mean value ± SD. *=p<0.05. (B) Relative frequency of CD133+CXCR4+ cells in invading cells fraction, recovered after treatment with CTCE-9908 and scramble peptide, compared to PDX total population (n=4 models in duplicate experiments. *=p<0.05. (C) Fold change in CD133 and CXCR4 positive cells in treated H460 xenografts compared to control, evaluated by FACS. Dara are mean % ± SD n=4 per group. (D) RT-qPCR for stemness genes expression in cisplatin or cisplatin+CTCE-9908 treated H460 xenografts. Untreated control tumors were used as calibrator. (E) Representative IHC staining for human cytokeratins of H460 lung metastases in control, cisplatin and cisplatin+CTCE-9908 treated groups.
10X magnification. (F) Percentage of CD133 and CXCR4 positive cells in H460 lung metastases and (G) in LT111 disseminated tumor cells (DTCs) in control and treated groups, as evaluated by flow cytometry. Data represent the mean value ± SD. n=4 mice per group. *=p<0.05, **=p≤0.01, ***=p≤0.001.

**Figure 4.** CD133\(^+\)CXCR4\(^+\) and EpCAM\(^-\) cells are crucial in early steps of colonization. (A) Time course analysis of i.v. injected LT111, LT73, H460 tumor cells. Arrow on the left indicates increasing metastatic behavior of tested tumor cells. Percentages of tumor cells (left Y axis) and relative fold increase in CD133\(^+\)CXCR4\(^+\) and cells CD133\(^+\)EpCAM\(^+\) subsets are evaluated by FACS analysis of murine lungs. Data represent mean ± SEM. n=2 mice at given time point. (B) RT-qPCR analysis for stemness genes expression of H460 cells, 2h, 1 and 3 weeks after i.v. injection in murine lung. H460 parental cell line was used as calibrator.

**Figure 5.** DTCs are enriched in CD133\(^+\)CXCR4\(^+\)EpCAM\(^-\) CICs and generate tumors with distinctive features. (A) Percentages of CD133\(^+\)CXCR4\(^+\)EpCAM\(^-\) subset within CD133\(^+\) cells in total PDX cells and in the corresponding invading cells fraction recovered after invasion assay, evaluated by FACS as shown on the right. n= 5 PDXs. p=0.01. (B) Representative image of lung spheroids culture and IF staining for human CKs on lung spheroids cytospin, identifying single human DTC (upper panel). Representative RT-qPCR amplification plot (lower panel) showing the increase expression of human β2microglobulin (B2M) gene in lung spheroids (red circle) compared to lung tissue of PDX-bearing mice (green circle). Mouse B2M endogenous control gene is equally expressed between samples. (C) Relative quantification of stemness genes (on the left) and EMT associated genes expression (on the right) in lung DTCs compared to corresponding PDX-CTOS, used as
calibrator for the analysis (n= 2 independent experiments). (D) H&E of PDX and corresponding DTCs-tumor, derived from s.c. injection of lung spheroids (20X and 40X magnification). (E) Flow cytometry analysis of LT59 tumor from CTOS and DTCs indicating the enrichment for CXCR4⁺EpCAM⁻ subset (red quadrant, lower dot-plots) within gated CD133⁺ cells (upper dot-plots). (F) Growth curves of secondary tumors from s.c. injection of 1x10⁵ LT59-CTOS and LT59-DTCs primary tumors cells. Data are the averages of tumors weight ± SD. n=6 mice per group. Tumor take is reported. (G) Percentage of lung disseminated tumor cells evaluated by FACS in mice bearing secondary LT59-CTOS and LT59-DTC tumors. n=4 mice per group. *=p<0.05. (H) Representative FACS analysis of lungs of mice injected i.v. with 1x10⁵ LT59-CTOS cells and LT59-lung spheroid cells, enriched in DTCs. Metastases from DTCs (mouse H2Kneg cells) were analyzed for CD133 and EpCAM expression.

Figure 6. Microenvironment stimuli generate disseminating CICs. (A) Percentage of CD133⁺ cells evaluated by FACS in parental LT73 cell line and in LT73-EMT cells. n=10 independent experiments. P=0.0001. (B) Relative mRNA expression of EMT associated genes and stemness genes in LT73-EMT cells, mesenchymal stem cells (MSC) and cancer associated fibroblasts (CAFs). CAF cells were used as calibrator for analysis. (C) Fold-change of CD133⁺CXCR4⁺ and CD133⁺CXCR4⁺EpCAM⁻ subsets within CD133⁺ cells in LT73 parental and LT73-EMT cell lines, detected by FACS. Mean ± SD. n=10 independent experiments. *=p<0.05 (D) Representative FACS analysis for one primary CTOS culture (on the left) and after TGFβ treatment (on the right), showing the enrichment for CD133⁺ cells (upper dot plots) and the relative increase in CD133⁺CXCR4⁺EpCAM⁻ subset (red quadrant, lower dot plots). (E) Representative FACS analysis for CD133 expression in LT73 cell line, in LT73-CD133neg cell line, untreated and treated with TGFβ and 5weeks after TGFβ
withdrawal. Expression of EpCAM within gated CD133+ cells is shown (lower dot-plots). (F) Mean % ± SD of de novo generated CD133+ cells in LT73-CD133neg cell line cultured with different CAF-conditioned media (CM). n=3 independent analysis. (G) Real Time PCR from EMT-genes expression in LT73 CD133neg cell line after treatments with CMs alone or in combination with CTCE-9908. Untreated cells were used as calibrator. (H) Growth curves of tumors generated by s.c. injection of 1x10^5 LT73-CD133neg cells treated with CAF-CMs. Tumor weights (mg) reported are the mean ± SD. n=8 per groups. *p<0.05. (I) Flow cytometry analysis of tumors in (H) for CD133+ cells and relative content of CD133+CXCR4+EpCAM- subset. Data represent mean ± SD. n=4 per group. (L) Percentage of disseminated cells detected by FACS in lungs of mice bearing tumors from LT73-CD133neg cells treated with CAF-CMs. Mean ± SD. n=4 per group. *p<0.05.

Figure 7. Disseminating CICs in primary NSCLC correlate with poor prognosis. (A) Percentages of CD133+CXCR4+EpCAM- cells in paired primary tumors and lymph node metastasis (n=17, p=0.006) detected by FACS analysis, as shown on the right: tumor cells are identified as CD45-CD34-CD31 negative cells and relative percentages of CD133+CXCR4+EpCAM- subset are evaluated within gated CD133+ cells. (B) Kaplan-Meyer estimates of overall survival and disease-free survival for 78 NSCLC patients according to presence of disseminating CICs (CD133+CXCR4+EpCAM-) within primary tumors.
Figure 1

A

B

Primary Tumors
Lymphnodes

3 months post injection

6 months post injection

Tumor cells in the lung (%)

CD133+CXCR4+
CD133-CXCR4-
CD133+/CXCR4+

Primary Tumors
Lymphnodes

CD133+CXCR4+
CD133-CXCR4-
CD133+/CXCR4+

3 months post injection

6 months post injection

Tumor cells in the lung (%)

CD133+CXCR4+
CD133-CXCR4-
CD133+/CXCR4+

3 months post injection

6 months post injection

Tumor cells in the lung (%)

CD133+CXCR4+
CD133-CXCR4-
CD133+/CXCR4+

C

CD133+/CXCR4+
CD133+/CXCR4-
CD133-/CXCR4-
Area of invasion

LT111-PDX
LT73
H460
H460

D

Area of lung invasion (%)

LT111
LT73
H460

E

LT111 CD133+/CXCR4+ metastasis

Mouse H2K
CXCR4

CD133
CD133

40X
40X

40X
40X

40X
40X

40X
40X

40X
40X

40X
40X
Figure 2

A

Human bone

CD34

B

CD133+/CXCR4+ bone metastasis

C

CD133+/CXCR4- cells

CD133+/CXCR4+ cells

D

Osteoid thickness

E

CD133+/CXCR4+ lung metastasis
Figure 3

A

B

C

D

E

F

G
Figure 4

A

**LT111-PDX**
- Total tumor cells
- CD133+/CXCR4+ cells
- CD133+/EpCAM- cells

Tumor cells in the lungs (%)
2 h 7 days 14 days 30 days 90 days

Fold increase in cell subsets

**LT73**
- Total tumor cells
- CD133+/CXCR4+ cells
- CD133+/EpCAM- cells

Tumor cells in the lungs (%)
2 h 30 day 60 days 90 days 120 days

Fold increase in cell subsets

**H460**
- Total tumor cells
- CD133+/CXCR4+ cells

B

**H460**
- 2h
- 1 week
- 3 weeks

Relative mRNA expression (tumor cells in the lungs)
Oct4 NANO2 SOX2

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Figure 5

A. CD133+ cells in total PDX cells and CD133+ cells in invading cells fraction.

B. Endogenous control mB2M and exogenous control hB2M.

C. mRNA expression levels relative to CTOS-PDXs for OCT4, NANOG, and SOX2.

D. PDX and DTCs-tumor stained with 7AAD/H2K mouse.

E. Flow cytometry plots for LT59 CTOS-tumor and LT59 DTCs-tumor.

F. Survival curves for LT59-CTOS and LT59-DTCs.

G. Box plots showing tumor cells in the lung for CTOS-tumor and DTCs-tumor.

H. Tumor cells in the lung for LT59-CTOS and LT59-DTCs stained with 7AAD/H2K mouse.
Figure 6

A

B

C

D

E

F

G

H

I

L

LT73 parental

LT73 EMT

Relative % of CD133+ subsets

CDH1

FN1

VM

SNAI1

ITGA6

NANOG

Oct4

CD133neg cell line

LT73

un-treated

+ TGFβ

5 weeks post TGFβ withdrawal

CD133neg cell line

Relative mRNA expression levels relative to LT73CD133neg Ctrl

SNAI2

FN1

VIMENTIN

LT73 CD133neg cell line

CD133neg cell line

Relative % of cell subsets

CD133

CD133+CXCR4-

CD133+CXCR4+EpCAM-

Lung Dissemination

Tumor cells in the lung (%)

Relative % of cell subsets

CD133

CD133+CXCR4-

CD133+CXCR4+EpCAM-

Ctrl

+CAF-CM1

+CAF-CM2

+CAF-CM3

+CAF-CM4

Lung Dissemination

Tumor cells in the lung (%)

Ctrl

CAF-CM1

CAF-CM2

CAF-CM3

CAF-CM4

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Figure 7

A

**CD133+CXCR4+EpCAM**

*Primary Tumors*  
![](CD45/CD34/CD31)  
![](CD133)  
![](EpCAM)  

*Lymph node metastasis*  
![](CD45/CD34/CD31)  
![](CD133)  
![](EpCAM)  

B

**OVERALL SURVIVAL (OS)**  

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<th>Months</th>
<th>38</th>
<th>35</th>
<th>23</th>
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<th>10</th>
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<th>39</th>
<th>32</th>
<th>24</th>
<th>17</th>
<th>14</th>
<th>present</th>
</tr>
</thead>
</table>

**p=0.05**

**DISEASE-FREE SURVIVAL (DFS)**  

<table>
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<th>35</th>
<th>26</th>
<th>19</th>
<th>13</th>
<th>12</th>
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</table>

**p=0.03**
Microenvironment modulated metastatic CD133+/CXCR4+/EpCAM- lung cancer initiating cells sustain tumor dissemination and correlate with poor prognosis

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