

Circulating tumor cells and epithelial, mesenchymal and stemness markers: characterization of cell subpopulations

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Abstract: Until now detection and numeration of circulating tumor cells (CTCs) were essentially used as a prognostic factor in cancer progression. To extend the role of these kinds of analysis, it seems necessary to improve analytical methods related to isolation and characterization of CTCs. Discrepancies between published results corroborates this requirement. In this review we suggest a combination of markers able to reach the goal. Moreover to improve the clinical utility of CTC analysis, particularly in the therapeutic follow up of the disease, epithelial mesenchymal transition (EMT) level of a global CTC population should be studied.

Keywords: Circulating tumor cells (CTCs); epithelial mesenchymal transition (EMT); stemness

Submitted Aug 13, 2014. Accepted for publication Sep 30, 2014.

doi: 10.3978/j.issn.2305-5839.2014.10.04

View this article at: <http://dx.doi.org/10.3978/j.issn.2305-5839.2014.10.04>

Introduction

Circulating tumor cells (CTCs) are the main vehicles of the metastatic relapse and therefore one of the principal keys to fully understand and counteract metastasis development. Despite their importance, CTCs are rare blood events and heterogeneous, including a variety of different sub-populations with dissimilar phenotypic and functional characteristics. Epithelial mesenchymal transition (EMT), a normal morphogenetic process is also involved in the genesis of CTCs. EMT is accountable for embryological steps like gastrulation, organogenesis of heart, musculoskeletal system, peripheral nervous system and is implicated in the cell plasticity phenomenon. More recently its role in diseased states was particularly delineated during primary tumor metastasis (1). Thus, CTC features may change during time, converting from one state to the other and vice versa. Normal and pathological EMT are not identical. Not all steps described in developmental EMT are necessary for the invasive phenotype to be established and a partial EMT is sufficient to give rise to initiating tumor cells (2). CTC population may thus comprise a subset of cells, potentially the most dangerous, with self-renewal, multipotency and tumor initiating capabilities called

circulating cancer stem cells (CSCs). This concept is similar to the CSC theory that describes a subset of cancer cells present in the primary tumor tissue which possesses the properties of normal adult stem cells (3). All these aspects clearly reveal the huge need for a CTC-detection approach based on the description of multiple antigens characterizing the different CTC subpopulations: epithelial, mesenchymal and stem-like.

CTC marker expression

At the single cell level, we can define a CTC on at least two criteria expression of specific proteins, e.g., epithelial cellular adhesion molecule (EpCAM), N-cadherin, CD44 and/or by expression of specific genes. In blood cells from cancer patients, when specific markers are detected, providing the cell is CD45 negative, we can infer that we have determined a foreign entity that generally should not be present in the peripheral blood and which most probably is a cancer cell. The three most well-known families of antigen characteristics that may be present alone or in different combination on CTCs are the epithelial, the mesenchymal and the stemness markers.

Epithelial markers/phenotype

Epithelial cellular adhesion molecule (EpCAM)

EpCAM is a cell surface glycoprotein highly expressed in epithelial cancer cells and at lower level in normal epithelial cells (4). Its presence on the surface of a cell found in the blood testifies of its foreign origin. This cell can be considered as a cancer cell provided it shows cellular histological features. EpCAM was at the starting point of detection and numeration of CTCs and was particularly developed by CellSearch system (Veridex, Warren, New Jersey, USA) (5). Yet expression of this protein is dependent on the stage of EMT and when downregulated it can be expressed together with N-cadherin and vimentin and could finally disappear as E-cadherin does. Numerous antibodies against EpCAM were used to target epithelial cancer cells. It is well known from the paper of Rao *et al.* that EpCAM expression is downregulated in many CTCs (6). If patients with undetectable CTCs by EpCAM based technology have a more favorable prognosis compared to those with detectable CTCs, there may be other patients with a bad prognosis. The latter could have undetectable CTCs due to EMT which down regulates epithelial marker expression. This is particularly true for EpCAM and cytokeratins (Cks). A recent publication of Lustberg *et al.* has described the heterogeneity of a new CTC subpopulation including cells that are EpCAM⁺ Ck⁺ and pan- hematopoietic marker CD45⁺. These cells have both epithelial and hematopoietic cells characteristics. However they are not cancer cells but rather are tumor associated macrophages. These kinds of circulating associated tumor cells should be taken into account in cancer patient prognostic. They could be identified by use of CD68 marker (7).

E-cadherin

E-cadherin is a component of adherens junctions and one of the hallmarks of epithelial cells. This molecule functions in close cooperation with the actin cytoskeleton and enables resistance to forces causing cell detachment. It is one protein among others that is implicated to support epithelial tissue architecture. Expression regulation of E-cadherin has a major role in the EMT progression. When a cell is going towards the mesenchymal state, expression of this protein is decreased (8). When cancer cells escape from primary tumor, E-cadherin can be inactivated or downregulated by different mechanisms. Its downregulation can be done through promoter hypermethylation, histone deacetylation, and more importantly, transcriptional repression (9). Major

E-cadherin repressors are Snail and Slug. Snail appears to be the most important E-cadherin repressor related to tumor progression. TGF β which both represses Id proteins (Inhibitor of DNA-binding proteins) and activates Snail family members explain the links between TGF β signaling, E-cadherin repression and EMT initiation (10).

Cytokeratins (Cks)

Cks are the largest and most diverse class of intermediate filaments which are cytoskeleton components. At least twenty different Cks can be expressed. They are markers of normal epithelial differentiation, but they can be used as diagnostic tool to detect different circulating cells of carcinoma. To reach this goal, antibodies must be able to recognize most of the epitopes. Epithelial cancer cells express Cks, K8, K18 and K19, and are used in Veridex technology both with EpCAM to numerate epithelial CTCs. They have also been used to identify disseminated tumor cells (DTCs). These types of markers are specifically expressed by cells with epithelial phenotype. However, when EMT is ongoing their expression is often downregulated (11). Cells leaving the primary tumor intravasate into the blood and lose their Ck expression. They escape to the detection. Joosse *et al.* demonstrated that the use of an anti-Ck antibody cocktail is enabled to track cells losing partial epithelial phenotype. These results suggest that CTCs showing mesenchymal state could have, among numerous Cks, a residual expression of some of them (12).

Zonula occludens (ZO)

ZO are proteins of tight junctions (ZO-1, ZO-2 and ZO-3) and like E-cadherin are involved in epithelial tissue architectural maintenance (13). ZO-1 and ZO-2 are critical to junction assembly and permeability (14). In the absence of ZO-1 and -2, cells fail to form tight junctions. ZO-1 links the transmembrane tight junction components to the actin cytoskeleton. ZO-1 downregulation is one the earliest modifications after induction of EMT (15).

Epithelial splicing regulator1 (ESPR1)

Alternative splicing involves numerous gene transcripts to generate complex protein expression pattern that in turn contributes to cellular identity. In pathophysiological conditions such as cancer, a high level of ESPR1 expression is the hallmark of epithelial phenotype. It was demonstrated that during EMT both ESPR1 and ESPR2 were downregulated. ESPR1 is a master regulator of the EMT process and its ratio against RBFOX2 is able to define the

epithelial character of a cell. ESPRs are among the most essential epithelial specific genes able to achieve a complete phenotypic cellular switch in EMT (16-19). When the described ratio is high a single CTC can be considered as being an epithelial one.

Outcome

After blood CTC enrichment, when at least two of the previous markers (EpCAM, E-cadherin, Cks, ZO-1, ESPR1) are evidenced at single cell level, this CTC is characterized as a differentiated epithelial cancer cell, provided mesenchymal and stem markers are not observed.

Mesenchymal-like CTCs

Differentiated epithelial carcinoma cells can be transformed into a mesenchymal state and during this process different degrees of EMT occur. Then it can be difficult to define a pure mesenchymal CTC subpopulation. However we can assert the mesenchymal character of CTCs on protein expression criteria reflecting EMT process. We also underline activation of biochemical pathways and regulation of altered metabolism (such as PI3K/Akt, Twist). The following markers should be considered:

N-cadherin

Adherens junctions have a major role in the shape organization of epithelial tissue. Phenotypic changes leading to epithelial sheet movements lean on their modifications. Among proteins of adherens junctions are the classic cadherins with E-cadherin, N-cadherin subfamilies. N-cadherin is expressed in many cells and particularly in mesenchymal cells. Therefore it was used as a marker of EMT. During this process E-cadherin is switched off while N-cadherin is switched on. Abnormal expression of N-cadherin associated to a dramatic decrease of E-cadherin is a hallmark of mesenchymal character of CTCs resulting from E- to N-cadherin switch (20). Loss of E-cadherin in tumor cells is due to methylation of its promoter or to overexpression of transcriptional repressors which target its promoter (21,22).

Vimentin

Vimentin is a protein expressed in mesenchymal cells and commonly considered as a marker of EMT. Its expression induces mesenchymal shape of cells and increases their mobility (23). Vimentin has been found in normal blood cells. However in their studies Lustberg *et al.* never

detected cells that are simultaneously positive for CD45 and vimentin. Thus vimentin cannot be considered as a hematopoietic marker. However it can be difficult on solely the level of vimentin expression to distinguish the mesenchymal from the epithelial phenotype. The degree of confidence of this marker to identify a mesenchymal cell has to be demonstrated (7).

Twist1

Twist1 is a basic helix-loop-helix transcription factor, involved in embryogenesis and reactivated in cancers leading to EMT. It is implicated in tumor development and progression. This transcription factor negatively acts on the E-box of E-cadherin. Its role in metastasis and diffusion of CTCs implicates more complex relations with oncogenic and anti-oncogenic proteins. The role in EMT was described by the Puisieux laboratory (24). There are two Twist proteins: Twist1 and Twist2 having different role *in vivo*. Twist1 was used to determine for the first time the presence of mesenchymal CTCs in early diagnosed breast cancers together with Akt2 and Pi3K (25).

Akt and Pi3K

Numerous signal transduction pathways can impinge on the essential regulation of E-cadherin for EMT. The oncogenic serine/threonine kinase AKT which is a downstream effector of the phosphatidylinositol 3' kinase (PI3K) has been described as a transcription repressor of the E-cadherin gene. These two elements of signaling pathways are associated with EMT and thus can be used to define mesenchymal cells (25,26). They are part of the PI3K/AKT/mTOR pathway which plays the major role in motility of cancer cells. AKT pathway is pivotal in EMT and has a nodal function for extra and intra cellular signaling pathways. Its regulation is dependant of PI3K (positive) and Phosphatase PTEN (negative).

Zeb1

ZEB1 (zinc finger E-box binding homeobox 1) is a DNA-binding transcription factor. It is a direct transcriptional repressor of E-cadherin. This protein is an activator of EMT and is implicated in reciprocal regulation with miR-200 family members and MYB protein (27). In a model EMT decision network, (miR-34/SNAIL) coupled with (miR-200/ZEB), Lu *et al.* explained the existence of a partial EMT state or hybrid epithelial/mesenchymal phenotype. They showed that (miR-200/ZEB) loop has three steady states: epithelial (high miR-200, low ZEB), mesenchymal

(low miR-200, high ZEB) and partial EMT (medium miR-200, medium ZEB). The latter state has specialized characteristics such as collective migration as seen in lymphatic vessels (28).

Alternative splicing proteins (FGFR2IIIc, Mena, p120 catenin)

Fibroblast growth factor receptor 2 (FGFR2) belongs to a family of transmembrane receptor tyrosine kinases. An alternative splicing event at the mRNA level produces either the FGFR2 IIIb (epithelial) or IIIc isoform (mesenchymal) (17). Mena is an actin regulatory protein. hMENA Δ v6 is the isoform derived from the hMENA alternative splicing program. hMENA Δ v6 is expressed in those cancer cells displaying EMT features and migratory behavior (29,30). Catenins are proteins that are linked to the cytoplasmic domain of transmembrane cadherins. Spliced variants predominate in epithelial versus mesenchymal cells. The mesenchymal p120 catenin isoform is induced during EMT (31).

Plastin-3 (PLS3)

As demonstrated PLS3, is able to induce EMT in colorectal cancer cells. Although this study was devoted to colorectal cancer cells this factor deserves to be included in markers of mesenchymal CTCs (32). Moreover it seems a putative clue to identify mixed epithelial mesenchymal cells.

Outcome

Pure mesenchymal CTCs as discrete entities are difficult to demonstrate. This is due to the evolution from epithelial to mesenchymal status. Detection of mesenchymal markers only helps to demonstrate the ongoing of EMT. However at the end of EMT, before mesenchymal epithelial transition (MET) starts these markers could define a pure population among cells with mixed phenotype (epithelial and mesenchymal characteristics). Some markers, particularly transcription factors, can be determined more easily by qRT-PCR than by immunohistological staining. When two mesenchymal markers are associated with epithelial or stem markers we can attribute a mesenchymal character to the examined cell.

Stemness-like CTCs

Numerous studies have suggested that CSCs with tumor initiation capacity might be derived from either somatic stem cells or differentiated progenitor cells. It has been supposed and in part demonstrated that only this cancer

cell subpopulation may lead to relapse or metastasis (33). If most cancer cells lack the ability to form a new tumor and if only the dissemination of rare CSCs can lead to metastatic disease, the main goal of CTC research must be to recognize and kill this circulating-CSC population. After identification and isolation of CSCs among CTCs, we should be able to fight more efficiently the residual cancer disease. To understand the origin of circulating CSCs two hypotheses can be proposed. Cancerous somatic stem cells undergoing EMT migrate from the primary tumor into the blood and can be called mesenchymal CSCs. The second possibility is that they may arise from fully differentiated cancer cells acquiring migratory properties due to the development of EMT pathways (34). Whatever is the way, the final endpoint of the phenomenon is the dissemination of CSCs, directed towards a niche, through intermediary cells showing mixed epithelial, mesenchymal and stemness characteristics. This could nicely explain the variety of surface markers and/or transcription factor expression found for initiating tumor cells in CTCs. Thus the diversity of CTCs having for origin EMT, which can be relevant in either CSCs or differentiated cancer cells, underlines the difficulty to establish a significant choice of identification markers. This complexity can be increased according to the organ where the tumor initially developed. During the EMT, cancer cells are endowed with stemness characteristics. This transformation leads to mesenchymal stem cancer cells. When they undergo the reverse MET they become epithelial stem cancer cells. To identify a CSC in the blood whatever it is (epithelial or mesenchymal) the following markers are particularly useful:

Aldehyde dehydrogenase-1 (ALDH1)

ALDH1 was described in 1% to 2% of normal breast epithelial cells. The first study showing ALDH1 expression in breast cancer tissue was made by Ginestier *et al.* (35). They provided the first demonstration of ALDH1 as a biomarker of breast cancer risk. ALDH1 has been shown to identify breast CSC properties *in vivo* and *in vitro*. Expression of this enzymatic protein can be evidenced by immunostaining and can be considered as a prognostic and predictive breast cancer biomarker. Only few cells having these characteristics are sufficient to grow into differentiated solid tumor in NOD/SCID mice. These results evoke the capacity of ALDH1 positive CTCs to metastasize. In a clinical trial aimed to detect CTCs in early breast cancer diagnosis, Barriere *et al.* demonstrated that out of 130 patients 49 were CTC positive, of which 17 were

ALDH1 marker positive. Thus, this study demonstrated that a stemness CTC population can be found in blood samples of breast cancer without metastasis (36).

CD44

CD44 is a cell surface glycoprotein which was implicated in cell migration and metastasis (37). Breast CSCs can intravasate in the blood and this subpopulation can be characterized by the expression of cell-surface markers CD44⁺/CD24^{low/-}/Lin⁻ (38). Contribution of CSCs to tumorigenesis was supported by Al-Hajj *et al.* who showed that the transplantation of as few as one hundred CD44⁺CD24⁻ cells from breast carcinomas was sufficient to grow a differentiated solid tumor in NOD/SCID mice. Otherwise CD44⁻/CD24⁺ tumor cells did not induce tumorigenesis even when 10⁵ cells were injected in animal models. It is intriguing that CD44⁺/CD24^{low/-} and ALDH1^{positive} cell populations do not necessarily coincide. It has been suggested that these proteins could detect epithelial versus mesenchymal characteristics of stem cells (39). Alternative splicing of CD44 is able to give rise to a plethora of isoforms. Among the latter, some of them can be more specific markers of CTC stemness and deserve to be more intensively studied. Effectively, CD44 is encoded by 20 exons. Among the latter, 10 of them can be regulated by alternative splicing and are named variants or v exons. The resulting isoforms are expressed only in proliferating cells and particularly in cancer. In colorectal cancer, CSCs express CD44v6. Its expression endowed CSCs with metastatic potential (40). This CD44v6 membrane marker seems to be one of the best for detection of CSCs.

Gangliosides (GD2, GD3 and GD1a)

Some glycosphingolipids are involved in cell growth and motility. It was demonstrated that Fuc-(n) Lc4Cer and Gb3Cer were drastically reduced whereas GD2, GD3, GM2, and GD1a were greatly increased in CSCs. These kinds of gangliosides can be used as markers of CTC stemness. The ganglioside GD2 (a glycosphingolipid) is associated to CD44^{high}CD24^{low} previously established as CSC-associated cell surface phenotype. In a similar manner, GD3 can be associated with stemness features but GD1a is still putative marker (41,42).

ABC proteins (xenobiotic extrusion pump proteins)

Recent studies have suggested that CSC phenotype is linked to high level expression of the ABCG2 transporter. It functions as a system able to extrude many xenobiotics and

particularly chemotherapy drugs. As CSCs are resistant to the latter, this chemoresistance suggests a close link between stemness and expression of the transporter. Effectively high expression of ABCG2 has been demonstrated in CSCs of lung, pancreas and retinoblastoma (43-45). These results make of the ABCG2 transporter a potential candidate as a marker of stemness.

Molecular characterization

Many problems remain to establish distribution of the different subpopulations among CTCs captured by technologies which have been described in numerous reviews (46,47). If cells are individually isolated, the approach will be different from the one considered for a total CTC population.

Isolated single circulating cancer cell

Immunostaining

On a single cell isolated from enrichment samples, multiple characterizations, even mutation research and genomic analysis can be done. The dielectrophoresis Array technology (DEPArray) enables to identify each entity of cell populations based on multiparametric fluorescence and bright field criteria (48). In such a system, we can establish belonging of a CTC to a subpopulation (epithelial, mesenchymal or stemness). A CTC has an epithelial phenotype if EpCAM, E-cadherin, pan CK, ESPR1 staining is fully positive provided CD45 and N-cadherin is negative. On the other hand a CTC has a prevailing mesenchymal phenotype if N-cadherin, vimentin staining is positive provided E-cadherin, ESPR1 have a low expression, CD45 being always negative. The stemness characteristic of a CTC is recognized when ALDH1, CD44^{high}/CD24^{low}, GD2 staining is positive. The high positivity of E-cadherin or N-cadherin distinguishes the epithelial or mesenchymal phenotype of the CSC. Of course we can choose more suitable markers depending on the origin of the primary tumor.

qRTPCR

Due to the capacity of DEPArray, qRTPCR on single cells can be used to define the phenotype of each isolated CTC. Single cell qRTPCR can be led according to the workflow published by Ståhlberg *et al.* (49). As an example, the ratio between Mena invasive/Mena epithelial or FGFR2IIIb/FGFR2IIIc can help to define the mesenchymal or the

epithelial nature of a CSC (16,17). In the same way expression of p120catenin, CD44 splice variants can be used

Total CTC population

Among CTC subpopulations, there are several phenotypes: epithelial, epithelial-mesenchymal, mesenchymal and mesenchymal stem. These subpopulations are not strictly distinct as there is a continuum between their different stages. To approach the tumor initiating potential of a CTC population the ratio of some splicing variants could evidence the role of these cells in metastasis. At the time of primary diagnosis, they can provide a new prognostic factor of the disease. The following spliced variant ratio should be used: Mena invasive/Mena epithelial or FGFR2IIIb/FGFR2IIIc and ESPR1/RBFOX2. Such a method requires the establishment of a standardization threshold able to appreciate the EMT degree. An attempt is ongoing by using modified human mammary epithelial cells when EMT is induced by tamoxifen (50).

Conclusions

Lethality of cancer is caused by metastatic diseases for 90% of cancers. Moreover, very small number of CTCs has probably potential to form metastasis. CTCs are extremely difficult to study, but they are of great importance for translational research in oncology as possible prognostic, predictive and especially as potential targets for novel therapeutic approaches. These subpopulations are of interest provided analytical tools are able to identify these types of cells. Thus, effective isolations and simultaneously therapy methods for CTCs should be improved. Numeration of CTCs has a prognostic significance but cannot individualize the cells with tumor initiating capacity. The major aim of CTC research is to isolate the initiating tumor subpopulations as they could recapitulate donor patient's tumor biology. We must acknowledge that due to the heterogeneity of CTCs similar to that of primary tumor, the choice of characteristic markers of CTC subpopulations is a complicated issue. Numerous scientific publications have developed studies often focused on one or two markers. In contrast others have established gene profiles, the interpretation of which, from the point of view of a clinician, is particularly difficult. This can be done by improvement of isolation technologies and by development of studies to target the degree of EMT in a total CTC population. Without these enhancements, the clinical utility

of CTC determination cannot be demonstrated.

Acknowledgements

Disclosure: The authors declare no conflict of interest.

References

1. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003;15:740-6.
2. Micalizzi DS, Farabaugh SM, Ford HL. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *J Mammary Gland Biol Neoplasia* 2010;15:117-34.
3. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. *Nature* 2001;414:105-11.
4. Litvinov SV, Velders MP, Bakker HA, et al. Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. *J Cell Biol* 1994;125:437-46.
5. Sieuwerts AM, Kraan J, Bolt J, et al. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. *J Natl Cancer Inst* 2009;101:61-6.
6. Rao CG, Chianese D, Doyle GV, et al. Expression of epithelial cell adhesion molecule in carcinoma cells present in blood and primary and metastatic tumors. *Int J Oncol* 2005;27:49-57.
7. Lustberg MB, Balasubramanian P, Miller B, et al. Heterogeneous atypical cell populations are present in blood of metastatic breast cancer patients. *Breast Cancer Res* 2014;16:R23.
8. Gall TM, Frampton AE. Gene of the month: E-cadherin (CDH1). *J Clin Pathol* 2013;66:928-32.
9. Peinado H, Portillo F, Cano A. Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol* 2004;48:365-75.
10. Peinado H, Quintanilla M, Cano A. Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J Biol Chem* 2003;278:21113-23.
11. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006;7:131-42.
12. Joosse SA, Hannemann J, Spötter J, et al. Changes in keratin expression during metastatic progression of breast cancer: impact on the detection of circulating tumor cells. *Clin Cancer Res* 2012;18:993-1003.

13. Lindley LE, Briegel KJ. Molecular characterization of TGFbeta-induced epithelial-mesenchymal transition in normal finite lifespan human mammary epithelial cells. *Biochem Biophys Res Commun* 2010;399:659-64.
14. Umeda K, Matsui T, Nakayama M, et al. Establishment and characterization of cultured epithelial cells lacking expression of ZO-1. *J Biol Chem* 2004;279:44785-94.
15. Runkle EA, Mu D. Tight junction proteins: from barrier to tumorigenesis. *Cancer Lett* 2013;337:41-8.
16. Warzecha CC, Shen S, Xing Y, et al. The epithelial splicing factors ESRP1 and ESRP2 positively and negatively regulate diverse types of alternative splicing events. *RNA Biol* 2009;6:546-62.
17. Warzecha CC, Carstens RP. Complex changes in alternative pre-mRNA splicing play a central role in the epithelial-to-mesenchymal transition (EMT). *Semin Cancer Biol* 2012;22:417-27.
18. Baraniak AP, Chen JR, Garcia-Blanco MA. Fox-2 mediates epithelial cell-specific fibroblast growth factor receptor 2 exon choice. *Mol Cell Biol* 2006;26:1209-22.
19. Hovhannisyanyan RH, Carstens RP. Heterogeneous ribonucleoprotein m is a splicing regulatory protein that can enhance or silence splicing of alternatively spliced exons. *J Biol Chem* 2007;282:36265-74.
20. Hazan RB, Qiao R, Keren R, et al. Cadherin switch in tumor progression. *Ann N Y Acad Sci* 2004;1014:155-63.
21. Li L, Bennett SA, Wang L. Role of E-cadherin and other cell adhesion molecules in survival and differentiation of human pluripotent stem cells. *Cell Adh Migr* 2012;6:59-70.
22. Khan MA, Chen HC, Zhang D, et al. Twist: a molecular target in cancer therapeutics. *Tumour Biol* 2013;34:2497-506.
23. Satelli A, Li S. Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cell Mol Life Sci* 2011;68:3033-46.
24. Ansieau S, Morel AP, Hinkal G, et al. TWISTing an embryonic transcription factor into an oncoprotein. *Oncogene* 2010;29:3173-84.
25. Barrière G, Riouallon A, Renaudie J, et al. Mesenchymal and stemness circulating tumor cells in early breast cancer diagnosis. *BMC Cancer* 2012;12:114.
26. Agarwal R, Carey M, Hennessy B, et al. PI3K pathway-directed therapeutic strategies in cancer. *Curr Opin Investig Drugs* 2010;11:615-28.
27. Hugo HJ, Pereira L, Suryadinata R, et al. Direct repression of MYB by ZEB1 suppresses proliferation and epithelial gene expression during epithelial-to-mesenchymal transition of breast cancer cells. *Breast Cancer Res* 2013;15:R113.
28. Lu M, Jolly MK, Levine H, et al. MicroRNA-based regulation of epithelial-hybrid-mesenchymal fate determination. *Proc Natl Acad Sci U S A* 2013;110:18144-9.
29. Pino MS, Balsamo M, Di Modugno F, et al. Human Mena+11a isoform serves as a marker of epithelial phenotype and sensitivity to epidermal growth factor receptor inhibition in human pancreatic cancer cell lines. *Clin Cancer Res* 2008;14:4943-50.
30. Di Modugno F, Iapicca P, Boudreau A, et al. Splicing program of human MENA produces a previously undescribed isoform associated with invasive, mesenchymal-like breast tumors. *Proc Natl Acad Sci U S A* 2012;109:19280-5.
31. Keirsebilck A, Bonn e S, Staes K, et al. Molecular cloning of the human p120ctn catenin gene (CTNND1): expression of multiple alternatively spliced isoforms. *Genomics* 1998;50:129-46.
32. Sugimachi K, Yokobori T, Iinuma H, et al. Aberrant expression of plastin-3 via copy number gain induces the epithelial-mesenchymal transition in circulating colorectal cancer cells. *Ann Surg Oncol* 2014;21:3680-90.
33. Sun S, Qiu XS. Cancer stem cells and tumor metastasis. *J Cancer Res Ther* 2013;9 Suppl:S150-2.
34. Liu S, Cong Y, Wang D, et al. Breast Cancer Stem Cells Transition between Epithelial and Mesenchymal States Reflective of their Normal Counterparts. *Stem Cell Reports* 2013;2:78-91.
35. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007;1:555-67.
36. Barriere G, Riouallon A, Renaudie J, et al. Mesenchymal characterization: alternative to simple CTC detection in two clinical trials. *Anticancer Res* 2012;32:3363-9.
37. Balic M, Lin H, Young L, et al. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin Cancer Res* 2006;12:5615-21.
38. Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 2003;100:3983-8.
39. Zhang Y, Toy KA, Kleer CG. Metaplastic breast carcinomas are enriched in markers of tumor-initiating cells and epithelial to mesenchymal transition. *Mod Pathol* 2012;25:178-84.

40. Todaro M, Gaggianesi M, Catalano V, et al. CD44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. *Cell Stem Cell* 2014;14:342-56.
41. Battula VL, Shi Y, Evans KW, et al. Ganglioside GD2 identifies breast cancer stem cells and promotes tumorigenesis. *J Clin Invest* 2012;122:2066-78.
42. Liang YJ, Ding Y, Lavery SB, et al. Differential expression profiles of glycosphingolipids in human breast cancer stem cells vs. cancer non-stem cells. *Proc Natl Acad Sci U S A* 2013;110:4968-73.
43. Ho MM, Ng AV, Lam S, et al. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res* 2007;67:4827-33.
44. Wang YH, Li F, Luo B, et al. A side population of cells from a human pancreatic carcinoma cell line harbors cancer stem cell characteristics. *Neoplasma* 2009;56:371-8.
45. Seigel GM, Campbell LM, Narayan M, et al. Cancer stem cell characteristics in retinoblastoma. *Mol Vis* 2005;11:729-37.
46. Joosse SA, Pantel K. Biologic challenges in the detection of circulating tumor cells. *Cancer Res* 2013;73:8-11.
47. Parkinson DR, Dracopoli N, Petty BG, et al. Considerations in the development of circulating tumor cell technology for clinical use. *J Transl Med*.2012;10:138.
48. Fabbri F, Carloni S, Zoli W, et al. Detection and recovery of circulating colon cancer cells using a dielectrophoresis-based device: KRAS mutation status in pure CTCs. *Cancer Lett* 2013;335:225-31.
49. Ståhlberg A, Kubista M. The workflow of single-cell expression profiling using quantitative real-time PCR. *Expert Rev Mol Diagn* 2014;14:323-31.
50. Barrière G, Fici P, Rigaud M. Dedifferentiated circulating tumor cells and epithelial mesenchymal transition in Breast cancer: From epithelial cell to the stem cells. International Stem cells symposium October 4, 2013. Faculté de médecine Pierre et Marie Curie, Paris. Available online: <http://www.fhf.fr/Actualites/Medecins/Agenda-medical/Colloque-international-sur-les-cellules-souches>

Cite this article as: Barriere G, Fici P, Gallerani G, Fabbri F, Zoli W, Rigaud M. Circulating tumor cells and epithelial, mesenchymal and stemness markers: characterization of cell subpopulations. *Ann Transl Med* 2014;2(11):109. doi: 10.3978/j.issn.2305-5839.2014.10.04