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EDITORIAL

Stem and immune cells in colorectal primary tumour: Number and function of subsets may diagnose metastasis

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Abstract

An important percentage of colorectal cancer (CRC) patients will develop metastasis, mainly in the liver, even after a successful curative resection. This leads to a very high mortality rate if metastasis is not detected early on. Disseminated cancer cells develop from metastatic stem cells (MetSCs). Recent knowledge has accumulated about these cells particularly in CRC, so they may now be tracked from the removed primary tumour. This approach could be especially important in prognosis of metastasis because it is becoming clear that metastasis does not particularly rely on testable driver mutations. Among the many traits supporting an epigenetic amplification of cell survival and self-renewal mechanisms of MetSCs, the role of many immune cell populations present in tumour tissues is becoming clear. The amount of tumour-infiltrating lymphocytes (T, B and natural killer cells), dendritic cells and some regulatory populations have already shown prognostic value or to be correlated with disease-free survival time, mainly in immunohistochemistry studies of unique cell populations. Parallel analyses of these immune cell populations together with MetSCs in the primary tumour of patients, with later follow-up data of the patients, will define the usefulness of specific combinations of both immune and MetSCs cell populations. It is expected that these combinations, together to different biomarkers in the form of an immune score, may predict future tumour recurrences, metastases and/or mortality in CRC. It will also support the future design of improved immunotherapeutic approaches against metastasis.

Key words: Colorectal cancer; Metastasis; Stem cells; Immune surveillance; Dendritic cells; Prognosis; Flow cytometry; Lymphocytes; Regulatory cells

Core tip: Metastasis relies on differentiation of some cancer stem cells in the primary tumour niche led by many micro-environmental signals. These signals include the participation of immune cell subsets such as tumour-infiltrating lymphocytes, dendritic cells and regulatory populations. Metastatic stem cells can be identified in the removed primary tumour. The study of the number and function of these immune

cell populations in parallel with metastatic stem cells (MetSCs) in the primary tumour, together with follow-up data of patients, will define the usefulness of specific immune and MetSCs cell population combinations. This can be combined with defining new biomarkers as future predictors of tumour recurrences, metastases and/or mortality in colorectal cancer.

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INTRODUCTION

Even when a primary tumour has been perfectly removed by surgery, at the moment of diagnosis tumour cells may have disseminated and established themselves in distant locations (metastasis). Metastasis accounts for the vast majority of deaths from cancer. Metastasis is a complex phenomenon developing through several stages, such as the intravasation of cancer cells from the primary tumour, dissemination through the circulation and extravasation in different organs, survival on arrival and settlement into latency, and reactivation, division and colonization of the organ, generating a new macroscopic tumour.

Migrant cancer cells that manage to settle in a distant tissue are known as disseminated tumour cells (DTCs)[1]. However not all DTCs are able to generate a new macroscopic tumour and those having such potential are called metastatic stem cells (MetSCs)[2]. The properties that support the survival, self-renewal, dormancy, and reactivation of these MetSCs have been recently reviewed[2], with the most remarkable conclusion being that MetSCs cells have been identified as are cancer stem cells (CSCs).

Most cancers display a hierarchical organization that resembles that of their tissue of origin. CSCs is the only cell type there with long-term self-renewal potential, the microenvironment niche sustaining this potential. They are the phenotypic and functional equivalent of normal stem cells but with the inconvenience of having acquired oncogenic mutations. Both CSCs and non-CSCs can display a migratory behaviour at the invasive front of primary tumours frequently associated with an epithelial to mesenchimal transition. MetSCs may derive from non-stem cell DTCs that reacquire the competence to initiate tumour growth after a period of latency, however this process of phenotypic plasticity is neither totally accepted nor well-understood[2-4]. However, the majority of extravasation and settlement survivors in the host tissue that endow tumour-initiating capacity (*i.e.*, MetSCs) are CSCs[5].

It can be deduced from the data above that MetSCs-cells harbouring the signaling pathways capable to initiate metastasis- already exist in the primary tumour and MetSCs can be tracked from the removed primary tumour. This approach would be specifically important for metastasis diagnosis since it is becoming clear that metastasis does not particularly rely on driver mutations. Therefore, genomic biomarkers are not actually useful for metastasis diagnosis. Environmental and tumour environmental signals do provide the epigenetic amplification for cell survival and self-renewal mechanisms[6].

METASTATIC STEM CELLS IN COLORECTAL CANCER

Colorectal cancer is the third most prevalent tumour worldwide. In developed countries, around a 30%-50% of patients who were through a successful curative resection still relapse or develop metastases, mainly in the liver. These patients show a very high mortality rate if those metastases are not detected early[7].

In CRC many lines of evidence support that MetSCs are already present in the primary tumour. A first line of evidence comes from marking of tumour cell populations with lentivirus, which has allowed the clonal analysis of human colorectal cancer (CRC) cells, showing that metastases arise from primary tumour cells that display long-term self-renewal capacity, are quiescent, and resistant to chemotherapy (*i.e.*, CSCs)[8,9].

A second line of evidence comes from experiments with genetic mouse models. Upon acquiring activating mutations in the Wnt pathway, intestinal stem cells generated adenomas[10]. Another lineage-tracing analysis showed that a stem cell population resembling those present in normal intestinal mucosa not only sustained the long-term growth of these benign lesions[11-13], but also of late stage CRCs and even liver metastases[14-16]. In mice, cell populations characterized by the expression of stem cell markers isolated from human primary tumour samples (CRC and other epithelial tumours) were capable of generating metastasis when transplanted[17-19]. The last line of evidence comes from the clinic, since high expression of adult stem cell markers in primary tumours have been associated with poor prognosis and metastatic relapse[14,15,18,19].

BIOMARKERS OF METASTATIC STEM CELLS

An important current question is which stem cell markers should be used for CSCs characterization and whether MetSCs are in fact a CSCs subset that can be tracked using present knowledge.

We have just demonstrated that soluble CD26 levels (sCD26) are a much better serum biomarker for the detection of CRC metastasis or tumour recurrence when compared to other markers in clinical use such as CEA, CA-19.9 or CA-72.4[7] levels. At the same time, others have demonstrated the relationship

between the presence of $CD26^+$ cells, detected by immunohistochemistry in primary CRC tumour biopsies and prognosis of metastasis[20]. It is plausible these results are related to the $CD26^+$ CSC population capable of generating metastasis when transplanted in mice[18]. This population comprised $CD133^+$, $CD44^+$ and $CD26^+$ cells isolated from the primary tumour. However, although they majorly encompass the known features of CSCs, they were not the only CSCs present in the primary tumour biopsies. In fact, due to plasticity in CD133 and CD44 expression, these markers do not seem the most appropriate at least as MetSCs markers[21-24]. Another candidates for CRC MetSCs characterization have been described including CD166, CD29, CD24, Lgr5, EpCAM, ALDH1, CDCP1, CXCR4, CC188[21,23] and ephrin type B receptor 2 (EphB2)[25], although many of these markers are also expressed in normal colonic stem cells (*i.e.*, Lgr5, ALDH1, or CD29) complicating the distinction between CSCs and normal stem cells. Despite this, most of these markers are co-expressed in the primary tumour, so it is expected that a particular biomarker combination can be used to identify MetSCs in CRCs. This will help to understand the function of these CSCs and identify new therapeutic targets as well as to play a significant role in clinical disease management[26]. From our present knowledge, CRC MetSCs should be found among the high-expressing Wnt targets $Lgr5^{++}$ and $EphB2^{++}$ cell population[25,27] also co-expressing $CD133^+$ and $CXCR4^+$, markers of a well known metastasic cell population with a recently discovered autofluorescent subcellular compartment[4]. This autofluorescence results from the accumulation of riboflavin in ATP-dependent ABCG2 transporter-coated vesicles exclusively located within the cytoplasm of cells across different human tumour types with CSC features[4]. It is possible that CD26, intriguingly related to some extent to the CXCR4/SDF-1 axis[28], could also be included among these markers.

METASTATIC TRAITS IN PRIMARY TUMOURS

As mentioned above, cell subsets with gene expression signatures to mediate dissemination, survival capability on arrival to distant organs, and entering a dormant state in many cases[2,6-8] before metastatic spreading, have been repeatedly identified in primary tumours[4,25-28]. These traits may be used to predict future relapses before dissemination.

However, (1) there is only a very small percentage of cancer cells with these properties; and (2) these cells are originated by the epigenetic amplification caused by many supporting pathways[2,6]. Little is known about these pathways despite its major clinical importance, since killing latent MetSCs by depriving them of that support seems the most attractive therapeutic approach.

A likely site for selection of metastatic traits in primary tumours is at the invasive front, the intersection of an advancing tumour mass and the surrounding stroma. Cancer cells at the invasive front of primary tumours are exposed to the stresses of invading surrounding tissue, of hypoxia, and of the immune surveillance. This complex milieu includes cancer-associated fibroblasts (CAFs), newly generated blood vessels[29], tumor-associated macrophages, myeloid progenitor cells, and blood platelets. Various stromal cell types produce cytokines such as Wnt, Notch, tumour necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), hepatocyte growth factor and hedgehog, which support the survival and fitness of CSCs[16,30-32]. Under selective pressure, these signals skew the heterogeneous cancer cell population towards a preponderance of clones primed for survival, self-renewal, invasiveness, migration, and the stress of infiltrating distant tissues (*i.e.*, future MetSCs).

In fact, it seems that when the stroma of a primary tumor is rich in cells and signals resembling those of a particular distant tissue, cancer cell clones selected in this primary tumour could be primed to thrive in that particular tissue[2,29,30]. For example, cells and signals in a colorectal gut tumour resembling the liver environment will induce metastasis of this CRC in the liver[33].

At the same time, some already cited tumour-derived soluble factors together with other signals such as VEGF, SDF-1, IL-10, and enzymes such as indoleamine 2, 3-dioxygenase or cyclooxygenase-2, or the adenosine pathway, are well known factors responsible for the expansion of induced-T regulatory T cells (iTreg) in tumour-bearing hosts[34-37] as well as for inducing the accumulation of immature dendritic cells (iDCs), which in turn promote the expansion of iTreg[38]. Both phenotypically and functionally, iTreg cells are distinct from natural Treg (nTreg) and accumulate both in tissues and peripheral blood of cancer patients. These iTreg are presumably responsible for the suppression of anti-tumour functions of immune cells migrating to the tumour site, thus promoting tumour escape from the host immune response[35].

IMMUNE SURVEILLANCE IN COLORECTAL CANCER

From the point of view of the three immune hallmarks of cancer stating that tumours (1) are able to thrive in a chronically inflamed microenvironment; (2) can evade immunorecognition; and (3) are able to suppress immune reactivity[39], CRC is particularly known for the many evidences connecting tumourigenesis and inflammation, such as the decreased incidence of tumours in individuals under non-steroidal anti-inflammatory drug treatment, the increased incidence of tumours in overweight patients, and its relationship with commensal bacteria. We have reviewed recently these facts altogether affecting inflammation both locally and systemically[40].

According to this activation of the immune system, cells of the innate immune system such as neutrophils[41],

macrophages[42], natural killer (NK) cells[43] or DCs[44] as well as cells of the adaptive immune system such as $CD4^+$ helper and $CD8^+$ cytotoxic T lymphocytes (CTLs)[45,46] accumulate in sites of CRC development. Although immune cells release inflammatory mediators (see above) with proangiogenic and prometastasic effects[47] to the reactive stroma, at the same time tumour-infiltrating lymphocytes (TILs) in CRC have been shown to inhibit tumour growth and are associated with improved prognosis[46-52].

The concept of cancer immunoediting[53] has been divided into three phases namely elimination, equilibrium and escape[54]. In the elimination phase or cancer immunosurveillance, immune cells detect and eliminate transformed cells but this elimination could be incomplete in which case some tumour cells remain either dormant or continue to evolve accumulating further changes that can modulate the expression of tumour-associated antigens (TAAs) or other factors that increase their fitness. During this time the immune system still exerts a selective pressure eliminating some transformed clones but if this elimination is again incomplete, the process results in the selection of tumour cell variants (MetSCs among them) which are able to resist, avoid or suppress the anti-tumor response, leading to the escape phase[54].

It has been shown that CRC induces an immunosuppression state, marked by reduced secretion in patients of several cytokines such as IFN-γ or TNF-α by monocytes/macrophages. As this immunosuppression was reversible after resection of the affected tissue[55], this data held the promise of immunologically targeting tumour cells, provided the mechanisms of immune escape and tumour-induced immune suppression are overcome.

T CELLS

As previously mentioned, human CRC tissue is infiltrated by a variety of immune cells often in the margins of the transformed tissue, in the invasive front. Several studies have characterized the lymphocyte infiltration of CRC and confirmed the concept of prognostic impact of these TILs[45,56]. In most cases, the lymphocytes infiltrating the cancer tissue, and most frequently the area along the invasive margin, are either $CD4^+$ and/or $CD8^+$ T cells[57].

Despite their low numbers, $CD8^+$ T lymphocytes infiltrating the neoplastic epithelium are positively correlated with longer disease-free survival time[52,56-58] and in fact, the density of T $CD8^+$ and $CD45^+$ lymphocyte infiltration was recently shown to have a better prognostic value than the classic tumor node metastasis classification factor[59]. Previous data have shown that these TILs have antitumor activity[60,61], and some TAAs have been identified as potential targets of cytotoxic $CD8^+$ T lymphocyte responses[60-62]. Later, T cell responses against mutated normal antigens such as those of the microsatellite instable (MSI) subgroup of CRC or the small subgroup of tumours with no signs of MSI but also with high mutational load were detected[50,63,64]. Coherently, the microsatellite instability-high phenotype present in 15% of early CRC confers good prognosis[50,65]. Therefore these so-called tumour-specific somatic mutations are potentially the best targets for adoptive T cell therapy, although there are many open questions like how many somatic mutations create suitable epitopes[66]. However, at the same time these tumours have clearly adapted to this immune pressure because many CRCs express no or reduced levels of HLA-I[67-69]. Although this is a classical mechanism of transformed cells to avoid the host immune response[70,71], there are conflicting results regarding CRC expression of HLA class I antigens as associated with poor prognosis[72], probably because NK cells are important effectors in the anti-tumour response against CRC (see below).

During an immune response $CD4^+$ T lymphocytes can differentiate into two broad phenotypic subtypes: T helper 1 (Th1) or Th2[73,74]. These two different subtypes secrete different types of cytokines, and consequently activate different types of immune responses. Th1 lymphocytes secrete IFN-γ and TNF-α, which produce the activation of CTLs, NK cells, macrophages and monocytes, all of which contribute to a cellular immune response that is effective against tumour cells. However, Th2 lymphocytes secrete a different set of cytokines such as IL-4, IL-5, IL-10 or IL-13, all of which deviate the response to a humoral immune response, and this kind of immune response is less effective at eliminating cancer cells[73-75]. A shift towards a Th2 response has been shown in CRC patients, with reduced levels of Th1 cytokines and normal or elevated levels of Th2 cytokines, an imbalance that becomes more significant the further the disease progresses[76-78], with levels of the Th1 cytokines having a prognostic value in terms of patient survival[73].

The mechanism through which CRC cells can shift the T cell immune response could be due in part to the secretion of cytokines that inhibit the development of Th1 responses, such as TGF-β and IL-10, either by the CRC cells themselves or CAFs[79]. Among the roles assigned to TGF-β in cancer development[79-85], it has been cited the inhibition of T lymphocyte proliferation and differentiation preventing naïve T cells from acquiring effector functions[86] and the inhibition of the ability of TILs to kill cancer cells as well as tumour-specific $CD8^+$ cytotoxic responses[87], although recently discovered stromal factors such as tumour-derived exosomes carrying death receptor ligands directly contribute to apoptosis of activated effector $CD8^+$ T cells[88]. IL-10 immunosuppresses TILs[89] but this immunosuppressive effect is mainly indirect and mediated by DCs and Treg lymphocytes (see below)[90,91].

In addition, although the role of other T helper populations, Th17 and Th22, in the development of CRC is still unclear, it seems that decreased Th17 and Th22 responses are associated with the development of CRC[92].

B CELLS

Many of the TAAs identified in CRC so far, potential targets of cytotoxic $CD8^+$ T lymphocyte responses, has been done by the identification of auto-antibodies present in the plasma of cancer patients compared to healthy donors, and although the clinical significance of those serologically-defined antigens still have to be demonstrated, several are attractive candidates for cancer vaccines[60]. Interestingly, antibody responses against some TAAs correlate with $CD8^+$ responses in those patients[61,62], supporting the idea that the immune response taking place in CRC patients requires coordinated $CD4^+$, $CD8^+$ and B cell responses, turning Th2 anti-tumour responses a not so negative factor as previously supposed[73]. However, tumour-infiltrating $CD20^+$ B cells (TIL-B) have being poorly investigated despite their described positive prognostic value[93]. Engagement of tumour-reactive B cells may be an important condition for generating potent, long-term T cell responses against cancer[94].

DENDRITIC CELLS

Dendritic cells are key antigen-presenting cells that play a central role in the induction of immune responses including anti-tumour responses[95,96]. It has been shown that CRC patients have DCs infiltrating the tumour mass or the surrounding tissue forming clusters with T lymphocytes[97] and that this infiltration seems to correlate with a better prognosis[98,99]. In fact, activated and matured DCs induce an antigen-specific response leading to T cell proliferation and differentiation into helper and effector lymphocytes[100].

However, CRC tumour cells are able to impair the function of these cells. *In vivo* tumour-infiltrating DCs show an immature phenotype[101] and iDCs presenting self-antigens to both $CD4^+$ and $CD8^+$ T cells induce tolerance in those lymphocytes[102,103]. In this direction, tissue culture media from CRC explants inhibits DC maturation with reduced levels of CD54, CD86, HLA-DR and CD83, and induces IL-10 secretion while inhibiting secretion of IL-12p70, factors that inhibit Th1 immune responses and probably protect the tumour from a potent immune response[104]. Moreover, as mentioned before, iDCs correlate with infiltration and the expansion of iTregs[35,103].

NK CELLS

NK cells play a major role in the immune response to CRC[59] and are a prognostic factor[105].

NK cells are typically defective in infiltrating solid tumors with only 30% of patients showing NK infiltration and with only a 9% with more than four NK cells, as it has been shown in a large cohort[106]. Tumour cells has several mechanisms to inhibit recruitment and activation of NK cells[107-109], but this fact does not have a direct effect on tumor progression *per se*[107] probably explaining why the presence of NK cells in combination with $CD4^+$ T lymphocytes in colorectal tumours had no detectable effect on the clinical course of the disease[43,106].

However, in CRC the infiltration of both NK cells and $CD8^+$ T cells was associated with prolonged patient survival in the same study, suggesting NK-$CD8^+$ cell crosstalk in the tumor microenvironment[106]. These data agree with the fact that *ex vivo* activation and expansion of both NK and CTLs followed by their intraveneous infusion in patients with stage Ⅳ colon cancer improved their quality of life[110], or the fact that one of the mechanisms of action of cetuximab, a monoclonal antibody against the epidermal growth factor receptor widely used for the treatment of metastatic CRC (mCRC), is antibody-dependent cell-mediated cytotoxicity, triggered by Fc-gamma-R on NK cells[59,111].

Of the utmost importance is the fact that NK cells play a crucial role in preventing recurrence[112] probably because they are able to target CSCs/MetSCs[113].

REGULATORY CELLS

Treg cells characterized by the expression of CD25 and the transcription factor Foxp3 are critical for the prevention of autoimmunity and the regulation of immune responses to foreign and self-antigens[114]. Adaptive iTreg, a distinct population from nTreg, accumulate in tissues and the peripheral blood of cancer patients. In many of those human cancers high densities of such Tregs in the tumor correlates with poor disease outcome[115].

However, they are associated with an improved survival rate of CRC patients[115,116], or other carcinoma with prominent inflammatory infiltrates (*i.e.*, certain types of breast cancer), despite iTreg contrasted functionality[117,118]. A hypothesis has been put forward to explain this apparent contradiction indicating that those $Foxp3^+$ Tregs infiltrating the tumour mass were already in the healthy colorectal tissue to suppress excessive inflammation and immune responses resulting from the commensal microflora[103,119].

It has been hypothesized that these cells posses a contextual plasticity controlled and driven by the tissue microenvironment[103]. The main question is which factors or signals in the microenvironment regulate Treg functions thereby preventing adverse effects of chronic inflammation or autoimmunity[120]. It seems that the cellular content of the CRC infiltrate do that by silencing the tolerogenic pathway of plasmacytoid DCs[121]. These cells, different to myeloid DCs, additionally promote tolerance and Treg differentiation and suppressor functions in the solid tumour presumably *via* the Nrp-1/semaphorin-4^a pathway (plasmacytoid DCs are one of the major sources of semaphorin-4^a), and the infiltrate would block this pathway[120]. Thus, it is important monitoring not only for the frequency but also for the functionality of iTreg in cancer.

In addition, the presence of other regulatory populations such as natural killer T (NKT) cells or Bregs can

not be excluded since the nature of the regulatory cell types that dominate in any given tumour is not totally understood[122,123]. The role played by regulatory type Ⅰ and Ⅱ NKT cells has been studied in syngeneic mice models of colorectal and renal cancer. In those models, having both type Ⅰ and Ⅱ NKT cells or neither of them, Treg depletion was sufficient to protect against tumour outgrowth, however in those mice lacking only type Ⅰ NKT cells, Treg blockade was insufficient to protect mice pointing to an important role played by type Ⅱ NKT cells in suppressing tumour growth[123].

HYPOTHESIS

An important reduction in the level of serum sCD26 in patients with non-metastasic CRC makes sCD26 a promising candidate for a future serum screening test[124]. We have previously suggested that these altered levels in CRC could be due to alterations in the number or frequency of lymphocyte populations expressing this biomarker[7,125].

We pretend to analyze by flow cytometry the expression of CD26 in the different leukocyte cell populations mentioned above that could be identified in primary CRC tissue biopsies. These analyses will be combined in parallel with the analysis of the known markers for MetSCs in cells of the same tissue[48,126]. All this information, together with the follow-up of the patients for up to 5 years, will help to define the usefulness of different cell population combinations, both immune and CSCs, and/or biomarkers. These combinations will assemble an immune score[59,101] that functions as predictor of future tumour recurrences, metastases and/or mortality in CRC. At the same time, this increased knowledge will support a better design of future immunotherapeutic approaches against metastasis.

Moreover, from a methodological point of view, the use of flow cytometry allows very potent qualitative and quantitative multiparametric analyses, contributing with new information to classical and modern[57,58,127] anatomopathological studies where no *in situ* information is lost.

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