Search for Theragnostic Biomarkers in Colorectal Liver Metastases: Focus on the Host Immune Cells

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ABSTRACT

The prognostic factors of patients with colorectal liver metastases (CLM) have been traditionally searched among the factors related to the primary colorectal tumor or to CLM. While many different scoring systems have been developed based on those traditional clinic-pathological factors, there has been the introduction of genetic biomarkers that added a theragnostic perspective. More recently, other important factors, such as those related to the host immune system, have been proposed as new determinants of prognosis of CLM patients. We herein review the current prognostic factors of CLM patients focusing on the burgeoning shifting paradigm of prognostication that relies on the host immune system.

Key words: host immune system, colorectal liver metastases, colorectal cancer liver metastases, colorectal cancer, immune response, immunotherapy

INTRODUCTION

Colorectal cancer is the third-fourth most common cancer as well as the second-third cause of cancer-related deaths worldwide (1). Being the liver the main filter of the venous drainage of the bowel, most of the patients with colorectal cancer develop colorectal liver metastases (CLM). As known, hepatic resection for CLM combined with systemic chemo-immunotherapy (SC) may be a curative therapy, as this approach has been associated with 5- and 10-year survival rates up to 50% and 35%, respectively (2). Of note, SC has increasingly been used as part of a multidisciplinary strategy not only to treat patients, but also to select the most eligible candidates for surgical resection of CLM (3-5). Innovative and effective SC regimens, including oxaliplatin-based and irinotecan-based therapies with or without biological therapies using anti-vascular endothelial growth factor (VEGF) or anti-epidermal growth factor receptor (EGFR) inhibitors have changed the natural history of CLM (6). This has led to the opportunity to offer hepatic resection to a significant proportion of patients with advanced tumor burden, who would not have been considered for surgery until few years ago.

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To date, different score systems showing heterogeneous results have been developed to calculate the prognosis of CLM patients undergoing hepatic resection (7-9). Nevertheless, in the era of precision medicine, there is still a strong need of new and reliable prognostic markers to cope with the known high heterogeneity of CLM that is responsible for a wide spectrum of clinical presentations and different degrees of responsiveness to therapies. Among the variables contributing to the different clinical outcomes of CLM, host immune responses certainly play a pivotal role (10-13).

Discovering the complex relations between CLM and the micro-environment represents one of the priorities in the understanding cancer biology. In this article, we review the prognostic factors for patients undergoing hepatic resection for CLM focusing on the emerging shifting paradigm that concerns the role of the host immune system.

Demographic prognostic factors

Generally, age and gender are tested as prognostic factors in clinical studies. Usually, gender does not significantly impact the outcome (2,14,15), while age may have more influence. Advanced age, in fact, may be associated with increased operative risk and consequently with postoperative complications. Patients with complicated postoperative courses as well as with decompensated comorbidities are unlikely to be able to complete the therapeutic program such as to start postoperative SC (16-19). Moreover, a recent interesting study on a large multicenter cohort of resected patients showed that age does not affect the long-term prognosis even if after 6 years from the hepatic resection the probability to be cure from this disease starts to increase for younger patients while starts to decrease for older patients. This was due to the onset of other age-specific causes of deaths (2).

Prognostic factors related to the primary colorectal tumor

The long-term prognosis of resected CLM patients may be impacted by many different factors related to the primary colorectal tumor. *Table 1* details such factors. Apart from T- status, N-status, and poor differentiation, which are two well-known prognostic factors (14,17-20), it is interesting to note that recently there was a countertendency about the location of the primary tumor. Historically, tumors in the rectum were thought to be the most aggressive (7,8,14), but

Table 1 - Traditional prognostic factors of colorectal liver metastases patients

Factor	Prognosis
Age (older versus younger)	+/-
Sex (male versus female)	=
Embryonic origin (midgut versus hindgut)	-
Advanced T-stage	-
Positive N-stage	-
Poor grading	-
Elevated CEA	-
Presence of extrahepatic disease	-
Elevated number of liver metastases	-
Size of liver metastases (larger versus smaller)	-
Synchronous versus metachronous disease	-

recently some studies underlined how tumors in the right colon may be the most aggressive (21). Indeed, it has been shown that the embryonic origin of the colon cancer (midgut versus hindgut) may be associated with different pattern of response to preoperative therapies and consequentially to different rates of survival. However, a note of caution should be considered when comparing such different studies, in which the proportion of synchronous CLM is different and the amount of missing data – specifically about the primary colorectal tumor data – is unknown. Yet, a true comparison would require at least a retrospective case-control study with a very large cohort of CLM patients with the same primary and secondary tumor burden submitted to the most similar systemic and loco-regional treatments.

Carcinoembryonic antigen

Even if disparate results are reported in the literature about its role, the level of carcinoembryonic antigen (CEA) is traditionally considered of importance both for the diagnosis as well as for the prognosis of CLM patients (7,22-25). Indeed, CEA level was found to be an independent factor correlated with 5-year overall and disease-free survival (23,24,26). As a matter of fact, CEA is routinely used in the daily clinical practice to monitor the presence of disease recurrence and to assess the response to SC (24,26). CEA represents the serum marker used as a surrogate of the biology of the disease. And, in the lack of more personalized and more sophisticated markers, it should be always tested.

Prognostic factors related to the colorectal liver metastases

Table 1 details the traditional prognostic factors of CLM patients. Tumor number, tumor size, bilobar

involvement, and status of the resection margin affect the survival (2, 14,15,27). Even if it is reasonable that these variables affect survival, they are of limited utility in the daily clinical practice. The use of only these variable does not allow to stratify the long-term outcome especially in the current era of modern and efficient SC (15,28,29). Similar considerations may be done for the histopathologic features, such as fibrous pseudocapsule formation, the degree of fibrosis around the metastases, single- versus confluent-node growing, infiltrative versus expansive type of growth, and invasion of intrahepatic vascular structures (30-32). All these features are important and informative, but they do not have an immediate translation in the daily clinical practice.

In general, metachronous CLM are associated with better outcome in comparison with synchronous CLM (14,15,33-35). However, many other studies reported similar outcome for synchronous and metachronous CLM (27,28,36,37). This contradictory result may be explained with different types of therapies, either systemic and loco-regional, and different adopted definitions of metachronous CLM. Notably, in another large multicenter cohort of resected CLM, synchronous CLM resulted to significantly and independently impact the overall and disease-free survival (2). However, synchronous presentation of CLMs should not affect the decision to operate but should influence the timing of resection, particularly in patients at high risk for recurrence meaning that perioperative SC should be always considered in those patients.

Surgical margin

It is noteworthy to affirm that the determination of margin width should be based on intraoperative ultrasound findings rather than on the liver palpation or just on what can be seen on preoperative images (38-40). Having said that, the surgical margin of CLM is always a subject of debate among experts. If microscopic negative margin (R0) is desirable in liver surgery, and it should be considered the primary endpoint in any liver resection, in the last years some works have supported that microscopic positive margin (R1), either parenchymal or vascular, may be oncologically adequate in some specific subgroups of patients (39,41,42,43). Conversely, other authors have showed that R1 resections should be avoided being associated with decreased survival (7,26, 44-48). In a large cohort of CLM patients, Pawlik et al. (49) reported that 1 mm of negative margin was enough to ensure a good outcome. Nowadays, experts agree that the biology of the disease rather than the

millimeter of the surgical margin dictates the prognosis of CLM patients (50). In regard to R1 resections, in particular in regard to R1-vascular resections that are the detachment of the tumor from major intrahepatic vessels, it should be noted that they have been found to be oncologically adequate as R0 resections (42,43). Moreover, those types of resections were conducted on patients otherwise marginally resectable because of advanced intrahepatic tumor burden. In other words, R1 resections may be better than no resections (42,43). Finally, it should be noted that the comparison among different CLM patients belonging to different studies is difficult. The differences in SC regimens, in intra- and extra-hepatic tumor burden, and in the statistical prognostic models used may, at least in part, explain the different conclusions (51,52).

Extrahepatic disease

The presence of extrahepatic disease, especially if multiple, represent not only a bad prognostic factor but also a contraindication for liver resection. As for the surgical margin, also for extra-hepatic disease the literature includes studies with disparate results. Elias et al. (53) showed that the number of CLM may be prognostically more important than the site of extra-hepatic disease. Conversely, other authors reported that the site of extra-hepatic disease should be taken into account (54). Yet, it is generally accepted that the presence of lung metastases is better than distant lymph nodes metastases (55). A given patient with lung metastases, even multiple, may be treated with wedge resections and achieve adequate survival (56-59).

RAS and RAF genes

KRAS, HRAS and NRAS, belong to the family of GTPases. When activated, KRAS can induce a cascade of mitogen-activated protein kinases (MAPKs) that transfers signals from the cell membrane to the nucleus. The RAS gene products activate proteins in the RAF family, which consists of the ARAF, BRAF and RAF-1 members (60). The importance around RAS mutations, rely on the possibility to predict resistance to the EGFR-targeted monoclonal antibodies. Such resistance may be seen in early stages as well as in advanced stages of the disease with an estimated prevalence up to 44% of CLM patients (61,62). Several authors have reported significant associations between RAS mutations and survival showing that its molecular determination should be nowadays part of the CLM patient evaluation (63-67). In regard to BRAF

mutations, they are reported as low as up to 2% of CLM patients (60). Nevertheless, has been showed that such mutations are strongly and independently associated with worse survival (60,68-70).

The role of the host immune system

Among the variables contributing to different clinical outcomes of tumors, the host immune responses certainly play a key role (10,11). Under homeostatic conditions human adult liver contains 10^{10} lymphocytes, with the majority of these cells being cytotoxic T and NK cells (71,721). Yet, others and we have reported that a highly functional and unique subset of NK cells physiologically resides in the hepatic sinusoids and these cells can comprise up to 50% of the total hepatic lymphocyte population (72,73). Hence, understanding the mechanisms inducing the infiltration of these anti-tumor cells into the CLM is highly relevant both for the estimation of the prognosis and for the development of novel and more effective therapies. A large amount of data that are available in the literature support that the infiltrating leucocytes have a major effect on the clinical outcome (13,74-78). The CLM patient is not an exception. Yet, the presence of tumor-infiltrating immune cells has been associated with prolonged survival in patients with colon cancer (12,13,79-83). Consistently, it is in fact well known that human liver is highly enriched of cytotoxic anti-tumor lymphocytes including T and NK cells that exert immune-surveillance against cancer (72,84,85). Recently, also our group has showed how the presence of intra-tumoral infiltration of NK cells was an independent prognostic factor favoring overall survival after resection of CLM (86). Those data and results support how the host immune system represents an emerging hallmark of cancer (87) which should not be any more left to exploratory translational researches while it should be

routinely considered in daily clinical practice. Indeed, in the last years the tumor microenvironment has been target of many studies aiming to find new promising therapies for solid tumors. Immune checkpoint inhibitors, such as ipilimumab and nivolumab have been introduced also for CLM patients with interesting findings (88). Yet, the understanding the biological mechanisms by which a given host immune contexture might be more favorable against colorectal cancer and CLM is the fundamental step that must be achieved to introduce innovative immunotherapies. More efforts should be done to identify which immunological factors are related to the tumors cells, which are related to the host, and how those factors interact to each other. Last not least, the role of loco-regional and SC in altering such factors should be one of the researchers' priorities. Yet, in large cohort of colorectal tumors, a gene-gene correlation network was built to understand which genes may be associated with anti-tumor response (89). As many as 65 different genes were identified, but interestingly most of them were linked to the host immune functionality (T and B cell activation, inflammatory response, T and B cells differentiation, adhesion- and migration-associated chemokines, activation of NK cells), which mean that certainly the immune system deserves further attention.

Table 2 details the immune intrigue that may be part of the liver tissue of CLM patients. Each of the cells represents a potential target for decoding the clinical heterogeneity of CLM patients, for developing new biomarkers, and consequently for developing new personalized immunotherapies. In line with this, some experimental data showed that the blocking of the EGFR pathway was associated with an increased immune infiltration in solid cancers (90,91). In this regard, several *in vitro* and *in vivo* models have shown that the masking of EGFR with a specific blocking monoclonal Ab (mAb) inhibits tumor proliferation,

Table 2. Immune intrigue of colorectal liver metastases patients

Cell type	Location	Role	Prognosis
T cell (CD4+ & CD8+)	Intra-tumoral; invasive margin	Specific tumor cell killing activity Release cytotoxic cytokines	+
Treg	Intra-tumoral; invasive margin	Suppress anticancer immune responses	-
Macrophage	Intra-tumoral; invasive margin	M1: tumor suppression and immune stimulation M2: malignant progression and suppressed CTL	+/-
NK	Intra-tumoral; invasive margin	Cytotoxic activity and cytokine production	+
MDSC	Intra-tumoral; invasive margin	Repression of the effector function of T lymphocytes and NK cells	-
DC	Intra-tumoral; invasive margin	Processing and presentation of tumor-associated antigens	+

Abbreviations: Treg, T regulatory; CTL, cytotoxic T lymphocytes; NK, Natural Killer; MDSC, Myeloid derived suppressor cell; DC; dendritic cells.

induces terminal cellular differentiation and modulates chemo- and radio-sensitivity (92). Recently, it has been also reported that the inhibition of the EGFR signaling pathway facilitates the activation of immune cells and their recruitment to tumor sites via the production of several cytokines and chemokines (90). This is particularly relevant for NK cells, as studies have shown that the use of a blocking anti-EGFR mAb stimulates these innate immune effector lymphocytes and induces antibody-dependent cell cytotoxicity (93,94). At the same time, the refractory effect of the tumor against this biological compound may be explained by the induction of mechanisms that the tumor can use to evade immune responses. Pre-clinical *in vivo* models have shown the existence of inducible mice carrying altered oncogenic and immunological pathways that are resistant to the inhibition of EGFR (95). Hence, since neo-adjuvant therapies in CLM adopt conventional chemotherapy agents (i.e. oxaliplatin and irinotecan), which are associated with biological drugs targeting different pathogenic signaling pathways in CLM, clinicians have to consider all possible mechanisms of oncogenic and immunological tumor escape in order to provide the most effective and customizable therapeutic options.

Hepatic macrophages as theragnostic biomarkers

Macrophages are immune cells that are widely distributed in the body. Their functions are the recognition, ingestion, and degradation of cellular debris, foreign material, and pathogens. Such functions are exerted by orchestrating the inflammatory response (96). They hold a central and important position into the liver during both acute and chronic liver injuries. This note relies also on one of the most important shifting paradigms about the origin of hepatic macrophages (97). Yet, it has been long believed that tissue macrophages originate from blood monocytes, but nowadays it has been demonstrated that there are many resident liver macrophages that persist independently from their blood counterparts (98). Moreover, considering that the liver harbors about 80% of all macrophages of the body plus that the liver is anyway patrolled by the blood monocytes lead to the considering them a central target for innovative therapies in liver diseases (99). To add further importance to these liver cells, it is important to remember that the modern radiological imaging of the liver relies exactly on the presence and function of the Kupffer cells, which are a subtype of liver macrophage (100). Of note, Kupffer cells and other subtypes of liver macrophages

cannot be easily distinguished from each other. Sophisticated multi-marker flow-cytometric phenotyping and intra-vital microscopy have been proposed in mouse (98). However, no single marker is currently able to discriminate these populations. Even, the traditional splitting of macrophages functions into inflammatory or “M1” versus anti-inflammatory or tumoral “M2” is nowadays under debate (101). This concept emerged from in-vitro experiments, but it may be too simplistic. In real conditions – especially in human diseases – macrophages are highly plastic and integrate multiple signals to shape their response (102). For instance, in the injured liver macrophages often express markers of inflammation or resolution simultaneously (103) and can rapidly change their phenotype depending on the hepatic microenvironment (104). Thus, much more efforts should be done to identify and characterize these important cells in relations to different diseases and different stages of the same diseases.

To date, no data are available on the role of tumor-associated macrophages (TAMs) in patients with CLM. Very few data are available for patients with hepatocellular carcinoma. In those last patients, the density of TAMs, evaluated by immune-histochemical staining for CD68, have been correlated with tumor progression and then with survival (105). Similar data have been recently proposed for patients with cholangiocarcinoma (106). Our research group is conducting a pioneering research on TAMs in CLM. Specific new subtypes of TAMs have been found in CLM patients with interesting correlations with clinic-pathological prognostic factors (unpublished data).

Taken together, the progresses in understanding macrophage heterogeneity in liver diseases, and in CLM patients, confirm the expectation that we will soon be able to translate these findings into novel therapies in clinical practice.

CONCLUSIONS

Survival following resection of CLM depends on several clinical, pathological, and molecular factors. Apart from those factors related to the tumor, both the primary colorectal and the CLM, there is the need to accurately consider also those factors related to the host immune system, which certainly plays an important role. Yet, the burgeoning data about the immunological intrigue of CLM patients deserve to be considered on a single-patient basis with the aim to be more sensitive in the prognostication as well as to introduce more efficient immune-therapies. Thus, the

sampling and analysis of the tissue microenvironment of CLM is essential and should be part of the standard histological examination of such patients.

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