

Hepatocellular Carcinoma: Methods of Circulating Tumor Cells (CTC) Measurements

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1. Introduction

Hepatocellular carcinoma (HCC) is responsible for significant morbidity and mortality in cirrhosis and also accounts for between 85% and 90% of primary liver cancer (Caldwell & Park 2009; Hussain & El-Serag 2009; Tandon & Garcia-Tsao 2009). Most of HCC in the world occur in the setting of cirrhosis and over half-million of people develop liver cancer every year and an almost equal number die of it (Caldwell & Park 2009; Hussain & El-Serag 2009). Liver cancer prognosis is determined by factors related to the tumor (etiologies) and factors related to the cirrhosis i.e. parameters of liver dysfunction (CLIP 1998; Llovet et al., 1999; Okuda et al., 1985; Tandon & Garcia-Tsao 2009). During the last 30 years the HCC-incidence rate increased dramatically, despite the development of the HBV-vaccine and the program for newborn vaccination against HBV, developed in European and Asian countries (El-Serag et al., 2003; Hussain & El-Serag 2009).

Since 1997, and after implementing a program for vaccination of newborns against HBV, Chinese and Japanese populations began to show a decrease in HCC incidence, especially among males (Plymoth et al., 2009; Yu S. Z. 1995). In the other hand, HCV-infection is rising around the world (Davila et al., 2004; Kong S. Y. et al., 2009; Min et al., 2009; Yoon et al., 2009; Yu M. L. & Chuang 2009) counter balancing the benefit effects of HBV-vaccination. This increase in incidence rate is observed through the world and it does not belong to a specific region. For example, the USA and Europe have the same positive incidence slope (Davila et al., 2004; Donato et al., 2006; El-Serag 2002; El-Serag 2004; El-Serag et al., 2003; Hassan et al., 2002; Hussain & El-Serag 2009; Scatton et al., 2009; Wu et al., 2000). The etiologies of HCC remain the same; the most important causes are the HBV and HCV infections, heavy alcohol consumption, aflatoxin B1, age and gender (males are more susceptible than females), race (Asian and African over 20 years old), tobacco consumption and obesity associated with non-alcoholic fatty liver disease and the increase of the Diabetes II mellitus that rise the risk factor between 2 and 3, genetic hemochromatosis, primary biliary cirrhosis, alpha1-antitrypsin deficiency and autoimmune hepatitis (Banks et al., 2006; Borgren et al., 1998; Bostick et al., 1998; Caldwell & Park 2009; CLIP 1998; Collier & Sherman

1998; Davila et al., 2004; Donato et al., 2006; El-Serag 2002; El-Serag 2004; El-Serag et al., 2003; Hassan et al., 2002; Hussain & El-Serag 2009; Idilman et al., 1998; Ishikawa et al., 1998; Jung et al., 1998; Llovet et al., 1999; Luzzi et al., 1998; Mendizabal & Reddy 2009; Mocellin & Hoon & et al., 2006; Mocellin & Keilholz & et al., 2006; Naume 1998; Naume et al., 1998; Okuda et al., 1985; Racila et al., 1998; Schutte et al., 2009; Wu et al., 2000; Yao D. F. et al., 2007; Yu M. C. et al., 2000; Yu S. Z. 1995).

Usually, HCC develops during a long process of inflammation and fibrosis, eventually leading to cirrhosis. (Britto et al., 2000; Hussain & El-Serag 2009; Idilman et al., 1998; McMahon 2009). The majority of liver masses are detected incidentally in asymptomatic patients. These lesions are identified using imaging tools such as ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI). A diagnostic approach to HCC has been developed based on the literature and expert consensus, and incorporates serology, cytohistology and radiology/imaging characteristics. (Assy et al., 2009; Bruix & Sherman 2005; Bruix et al., 2001; Byrnes et al., 2007; Durand et al., 2001; Gomaa et al., 2009; Hussain & El-Serag 2009; Mendizabal & Reddy 2009).

HCC is one of the most aggressive cancers. Patients who show progress over the terminal stage have a 1-year survival of less than 10%. In the other hand, patients presenting as early-stage HCC with preserved liver function, a solitary HCC or up to three nodules, each less than 3cm have 5-years survival figures up to 75%. The choice of the therapy and the prognosis are dictated by the severity of the liver function, portal hypertension and medical co-morbidities. National and international consensus were established to choose the best treatment adapted for each case and obtain the best prognostic. Milan, Barcelona Clinic Liver Cancer, Cancer of the Liver Italian Program, and San Francisco criteria had determined the prognosis of patients based on the number and size of nodules, the metastasis and the state of liver function leading to the best choice for the patient (Banks et al., 2006; Borgen et al., 1998; Bostick et al., 1998; Caldwell & Park 2009; CLIP 1998; Collier & Sherman 1998; Davila et al., 2004; Donato et al., 2006; El-Serag 2002; El-Serag 2004; El-Serag et al., 2003; Freeman R. B. et al., 2006; Hassan et al., 2002; Idilman et al., 1998; Ishikawa et al., 1998; Jung et al., 1998; Llovet et al., 1999; Luzzi et al., 1998; Naume 1998; Naume et al., 1998; Racila et al., 1998; Sanchez Antolin et al., 2009; Yao D. F. et al., 2007).

Based on these data, the physician can choose resection, orthotopic liver transplantation (OLT), percutaneous ethanol injection or radiofrequency ablation, chemoembolisation, systemic chemotherapy and symptomatic therapy. The first 3 treatments are potentially curative, the second following treatments are palliative and the last one usually is applied at the terminal stage of the disease (Mendizabal & Reddy 2009). At the time the HCC will be treated an important question that the physician has to face is the risk of recurrence and metastasis. To answer this question the best approach would be able to detect the circulating tumor cells in the bloodstream.

2. Definition of CTC and HCC

In the field of biology of tumors, some expressions have been coined for the different types of circulating cellular elements. The term *circulating tumor cells* (CTC) defines specifically the tumor cells detected in blood or lymphatic vessels. Circulating cells in the bloodstream or in

the lymphatic system are considered to be tumoral microemboli (CTM) and represent a collective migration. The terms disseminated tumor cells (DTC) and isolated tumor cell (ITC) can be also found in the literature, but are usually used to define the cells that can be detected in both the organs and the bloodstream. The word micrometastasis is usually used to indicate tumor cells found in distant organs. (Luzzi et al., 1998; Schuler & Dolken 2006; Zieglschmid et al., 2005). The presence of circulating tumor cells reflects the aggressiveness nature of a solid tumor. Many attempts have been made to develop assays that reliably detect and enumerate these cells. The clinical results obtained with such assays suggest that in some tumor types, CTC detection and identification can be used to estimate prognosis and may serve as an early marker to assess anti-tumor activity of treatment. In addition, CTC can be used to predict progression-free survival and overall survival. CTC are an interesting source of biological information in order to understand dissemination, drug resistance and treatment-induced cell death. (Alix-Panabieres et al., 2008; Braun et al., 2005; Curry et al., 2004; Mocellin & Hoon & et al., 2006; Mocellin & Keilholz & et al., 2006; Muller et al., 2005; Nakagawa et al., 2007; Pantel et al., 2008; Pantel & Woelfle 2005; Paterlini-Brechot & Benali 2007; Riethdorf et al., 2008; Sleijfer et al., 2007; Strijbos et al., 2008; Willipinski-Stapelfeldt et al., 2005).

In general, intra- or extrahepatic metastases appear at a late tumor stage or after treatment suggesting that earlier during the development of the HCC, the tumor spread circulating of liver-derived cells or circulating tumoral cells also named in literature "micrometastasis". In HCC animal models showed that 10 to 10 000 CTC are capable to initiate new metastasis (Groom et al., 1999; Hermanek et al., 1999; Liotta et al., 1974; Luzzi et al., 1998). Even after curative resection, the tumor recurrence rate remains high. Although CTC detection has been applied and well documented in different types of cancer, especially breast cancer, CTC detection is not routinely performed in HCC follow-up and remains in the experimental field. However, CTC detection might bring new interesting information of metastatic process, might be used as diagnostic tool of early recurrence and may allow a better patient selection for liver transplantation. Mechanisms of tumor recurrence are still poorly understood. Several arguments point out that HCC tumor cells can infiltrate the blood system as shown by the presence of alpha-fetoprotein mRNA (Aselmann et al., 2001; Kamiyama et al., 1996; Kienle et al., 2000; Komeda et al., 1995; Lemoine et al., 1997; Matsumura 2001; Matsumura et al., 2001; Matsumura et al., 1995; Matsumura et al., 1994; Matsumura et al., 1999; Morimoto et al., 2005). CTC seem to be correlated with poor survival in many types of tumors (Aselmann et al., 2001; Kamiyama et al., 1996; Komeda et al., 1995; Lemoine et al., 1997; Matsumura et al., 1994; Morimoto et al., 2005).

However, HCC circulating cells are still difficult to detect, and their presence and amount are poorly correlated with either long-term survival or recurrence in the setting of HCC. Given the lack of specific biological markers, few studies focused on CTC detection. The challenge of CTC detection is related to the requirement of both high sensitivity and specificity. A wrong labeling of "non-tumor cells" (epithelial non tumor cells or normal hepatocytes, for instance) as "tumor cells" could generate poor clinical interest.

Methods of CTC detection have to be highly sensitive and specific. The first technical challenge in this field consists of finding exceptional cells. Just a few CTC are mixed with the approximately 10 million leukocytes and 5 billion erythrocytes in 1 ml of blood. To be

useful, the method used to identify circulating tumor cells must also detect all tumor cells and discriminate them from non-tumor cells. (Alix-Panabieres et al., 2008; Becker et al., 2005; Braun et al., 2005; Curry et al., 2004; Mocellin & Hoon & et al., 2006; Nakagawa et al., 2007; Paterlini-Brechot & Benali 2007; Ross et al., 1993; Schuler & Dolken 2006; Smerage & Hayes 2006). Before considering the technical problems, it is important to have circulating hepatoma-specific biomarkers to be able to detect the CTC and further to be useful to early diagnosis, monitoring metastasis or post treatment recurrences of HCC.

Currently, CTC detection is mainly based on alpha-fetoprotein (AFP) messenger RNA (mRNA) assessment or quantification, and in few reported cases using cytomorphometric technology, especially the ISET devise. A high sensibility could be obtained using flow cytometry assays but high blood volumes (200 ml) and long analysis time (40 h for one sample) are required. Consequently, its use has been discouraged as a routine technique (Mejean et al., 2000).

3. HCC markers and their application for CTC

The hepatocellular carcinomas can synthesize various tumor-related proteins, polypeptides and isoenzymes more or less specific of the hepatoma tissues. It is important to detect CTC in the bloodstream or lymphatic system to have tumor specific markers of HCC. Finding one of these circulating proteins does not mean that there circulating tumor cells.

3.1 Secreted proteins

3.1.1 Relevance of cytokines in HCC circulating tumor cells

Hepatocellular carcinoma tumor has shown to secrete a lot of cytokines related to the development of the tumor, like vascular endothelial growth factor (VEGF), transforming growth factor-beta 1 (TGF- β 1), Interleukin 8 (IL-8) or tumor-specific growth factor (TSGF). These serum markers are useful to follow-up the development and the prognosis of the HCC but useless to follow-up circulating tumor cells in blood (Zhou et al., 2006).

3.1.2 The case of the serum AFP

The serum α -fetoprotein (AFP) levels show high levels in newborns and then declines progressively below 10 ng/ml in 300 days of life. An increase of serum AFP levels can be observed during pregnancy, and in patients with mucoviscidosis, acute hepatitis (30%-50%), chronic hepatitis (15%-50%), cirrhosis (11%-47%) and other cancers (gastrointestinal, pancreatic, biliary, non-seminomatous germ-cell testicular, and germ cell ovarian). Serum level AFP sensitivity is between 39% to 64% with approximately 60% for a cutoff of 20 ng/mL, and decrease to 22% if higher cutoff of 200 ng/mL is used (Bruix et al., 2001; Collier & Sherman 1998; Fujiyama et al., 2002; Okuda 1986; Saffroy et al., 2007; Trevisani et al., 2001). AFP specificity is around 76%-91% with a low predictive value between 9%-32% (Bruix et al., 2001; Collier & Sherman 1998; Okuda 1986; Saffroy et al., 2007).

In addition, the serum AFP level is correlated with the tumor size. 80% of small HCCs (<2cm) do not express AFP. In the other hand, AFP level can be elevated in patients with chronic liver disease with high degree of hepatocytes regeneration such as HCV-infection

that show a high level of AFP in absence of malignancy (Toyoda et al., 2004; Vejchapipat et al., 2004). For these reasons, some additional serological markers used in combination with AFP seem to improve the performance of this biomarker, especially in terms of sensitivity.

Studies were done by using one or two more markers like des- γ carboxyprothrombin (DCP) also called prothrombin induced by vitamin K absence II (PIVKAII) and glycosylated AFP-L3 (*Lens culinaris* Agglutinin-Reactive AFP) fraction serum levels to diagnose earlier HCC and increasing the sensitivity especially when HCC is associate with cirrhosis, HCV or HBV infection (Dohmen et al., 2003; Fujiyama et al., 2002; Gonzalez & Keeffe 2011; Lok et al., 2009; Min et al., 2009; Saffroy et al., 2007; Suehiro et al., 1994). But there is no study that correlates their serum levels and the circulating tumor cell during the HCC development.

3.2 The mRNA markers

In the next sections, we focus on and describe the most specific markers of messenger RNA used to detect CTC in hepatocellular carcinoma.

3.2.1 Relevance of α -fetoprotein (AFP) messenger RNA (mRNA)

Initially, the albumin gene has been proposed as a biological marker to track CTC during HCC development but has been rapidly abandoned since many groups have reported illegitimate transcription of albumin gene in peripheral-blood leukocytes. The relevance of AFP mRNA as a marker of circulating tumor cells is better but is also controversial because these cells have not been further characterized and it has been shown that they may correspond to normal circulating hepatocytes (Lemoine et al., 1997; Louha et al., 1999; Minata et al., 2001). Furthermore, these tumor cells have mostly been sought and detected shortly after liver resection (Kienle et al., 2000; Lemoine et al., 1997; Louha et al., 1999; Matsumura et al., 1994; Minata et al., 2001). This finding suggests that CTC could spread following liver mobilization or manipulation. Although the mechanisms leading to intra and extrahepatic recurrences are still unknown, some observations suggest that bone marrow (BM) could also be a specific reservoir of CTC. Indeed, several reports have suggested that tumor cells are of BM origin (Agarwal et al., 2004; Houghton et al., 2004; Sell 2002). Hepatic tumor stem cells may take advantage of the potential for stem cell support of the BM microenvironment. The amplification of AFP mRNA by means of reverse transcription (RT) and a nested polymerase chain reaction (PCR) is the highly sensitive method for the detection of residual HCC cells in peripheral blood. The qualitative (positive versus negative) detection of HCC circulating tumor cells in blood samples from individual patients is of limited value in predicting the risk of disease progression. Because the level of AFP mRNA is increased in HCC tissue compared with in normal hepatocytes, the quantification of AFP transcripts seems to be a more reliable indicator of disease progression. A more highly sensitive assay based on TaqMan® technology to quantify AFP mRNA in “real time” should be preferred (Cheung et al., 2006; Gross-Goupil et al., 2003; Lu Y. et al., 2007; Matsumura 2001). Even using this methodology, reported results are not homogeneous and contradictory (Bruix et al., 2001). The main studies which have evaluated AFP mRNA are summarized in Table 1. The false-positive results can be obtained using AFP mRNA.

Author	PCR	Sensitivity	Cases	Samples	Positivity	Predictability of recurrence
(Kamiyama et al., 1996)	qRT-PCR	1 CHC/ 10 ⁷	37 136	Blood BM	18% 28%	No Yes
(Morimoto et al., 2005)	qRT-PCR	1 CHC/10 ⁶	38 25	Blood BM	10 % 48%	Yes No
(Kienle et al., 2000)	nRT-PCR	5 cells/ 1mL	24	Blood BM	29% 43%	Suspect
(Aselmann et al., 2001)	Competitive RT	10 cells/ 9 mL	22 11	Blood BM	26% 45%	NA
(Sutcliffe et al., 2005)	RT-PCR	NA	18	BM	93%	No
(Matsumura et al., 1995; Matsumura et al., 1994)	nRT-PCR		33	Blood	52%	Extrahepatic metastases
(Komeda et al., 1995)	nRT-PCR	15 cells/mL	64	Blood	36%	Extrahepatic metastases
(Lemoine et al., 1997)	nRT-PCR	1CHC/10 ⁵ mono	20	Blood	25%	No
(Miyazono et al., 2001)	nRT-PCR	10 ⁻⁶ µg/µLof RNA	33	Blood	54%	Yes
(Ijichi et al., 2002)	nRT-PCR	1CHC/10 ⁵ mono	87	Blood	36%	Yes
(Witzigmann et al., 2002)	nRT-PCR	1 CHC/10 ⁷ mono	85	Blood	26-45%	No
(Gross-Goupil et al., 2003)	RT-PCR	1 CHC/10 ⁶	52	Blood	25%	No

Table 1. Evaluation of serum alpha-fetoprotein as a marker of circulation tumor cell in different hepatocellular carcinoma studies. nRT-PCR, nested RT-PCR; qRT-PCR, quantitative RT-PCR.

3.2.2 Transforming growth factor beta-1 (TGF-β1) mRNA

The levels of circulating TGF-β1 and TGF-β1 mRNA were significantly higher in the HCC patients than any other group of patients. The sensitivity and specificity of circulating TGF-

β 1 level ($>1.2 \mu\text{g/L}$) were 90% and 94% for HCC diagnosis, but no significant correlation was found between TGF- β 1 expression and AFP levels or tumor size. The combined detection of TGF- β 1 and serum AFP could raise the detection rate of HCC up to 97%. Both of circulating TGF- β 1 and TGF- β 1 mRNA could be used as sensitive biomarkers for diagnosis and prognosis of HBV-induced HCC (Dong et al., 2008; Wang Y. L. et al., 2007). Unfortunately, TGF- β 1 mRNA was poorly studied and further investigations have to be done to use circulating TGF- β 1 mRNA as a marker of circulating tumor cells in HCC.

3.2.3 Insulin-like growth factor (IGF)-II mRNA

Studies using amplified fragments of IGF-II mRNA by RT-PCR showed that the lowest sensitivity with 2 ng/L of total RNA. Dong et al., showed that the positive frequencies of IGF-II mRNA were 100% in HCC, around 50% in paracancerous and 0% in noncancerous tissues respectively. But, the positive frequency of circulating IGF-II mRNA was 34% in HCC, and no amplification was found in other liver diseases, extrahepatic tumors, and normal control, meaning that IGF-II is specific of the HCC but not really sensitive. Associated to other circulating markers IGF-II can be helpful to detect CTC. The circulating IGF-II mRNA was correlated with the stage of HCC (incidence=100%) with extrahepatic metastasis, and 35% with AFP-negative. No difference was found between tumor size and circulating IGF-II mRNA (Dong et al., 2005; Wang Y. L. et al., 2007) but these results are controversial (Qian et al., 2010).

3.2.4 Alpha-albumin (ALF) mRNA

For more than a decade, we know that mRNAs of hepatocyte-specific albumin genes are detected in peripheral blood by RT-PCR. It was shown that there is evidence that detection of albumin mRNA associated with the detection of AFP mRNA is strongly associated with the presence of metastases (Hillaire et al., 1994; Kar & Carr 1995; Komeda et al., 1995; Matsumura et al., 1995). Wong et al., showed that circulating hepatocellular carcinoma cells can be detected and be semi-quantified by albumin RT-PCR (Wong et al., 1997). On the other hand, Wu et al., showed that, the down regulation of alpha-albumin (ALF) specifically in HCC circulating cells can be used as a specific marker to discriminate the normal hepatocellular circulating cells that express abundantly ALF. RT-PCR ALF in association with RT-PCR AFP have been proposed to distinguish normal or malignant hepatocytes in peripheral blood, but the interpretation of the results is still debated (Resto et al., 2000).

3.2.5 Prostate-specific antigen (PSA) and mRNA PSA

The Prostate-Specific Antigen (PSA) had shown to be well-established reliability marker and remained a valid prostate marker in patients with acute hepatitis and HCC (Malavaud et al., 1999). But these results are controversial, PSA and mRNA PSA seem to don't be specific to the tissue and frequently detected in peripheral blood cells from healthy patients (Ishikawa et al., 1998). In addition, like the cytokines, serum PSA cannot be used as hepatocellular carcinoma marker for circulating tumor cells.

3.2.6 Heat shock protein (HSP)

Heat shock proteins (HSP) are stimulated under perturbation or stressors by the tissue. HSP are ubiquitous molecules and can be also expressed during carcinogenesis. Different HSP

have been related to the development of the hepatocellular carcinoma like gp96 or GRP94, HSP70 and HSP27, but none of them were used as a specific marker of circulating tumor cell (Wang Y. L. et al., 2007).

3.2.7 Human telomerase reverse transcriptase mRNA or hTERT mRNA

Human telomerase is a ribonucleic protein composed by the association of three structures: human telomerase RNA component (hTERC); human telomerase-associated protein 1 (hTEP1); and human telomerase reverse transcriptase (hTERT). hTERT is the catalytic unit of the complex. Also, telomerase is expressed in embryonic cells, in most human cancer cells or immortal cell lines, but not in normal somatic cell lines or tissues. For these reasons, hTERT was investigated as a marker of diagnosis and prognosis of HCC, but the results are controversial and appear that false-positive results can be observed because of lymphocytes, precancerous liver parenchymal cells and micrometastasis maybe responsible (for review (Grizzi et al., 2007; Wang Y. L. et al., 2007; Zhou et al., 2006)). Recently, Kong et al., investigated hTERT in peripheral blood in HCC from 343 Korean patients. There is no association between hTERT expression and clinical features and nor relationship between AFP and hTERT mRNA. Their conclusion is that AFP and hTERT mRNA expression in peripheral blood is useless as HCC prognostic markers (Kong S. Y. et al., 2009).

3.2.8 Cancer-testis antigens (CTA)

Cancer-testis antigens (CTA) represent a category of tumor-associated antigens normally expressed in male germ cells but not in adult somatic tissues (Scanlan et al., 2002). CTA are heterogeneous group of antigens. Actually, more than 44 distinct CT "gene" or "antigen" families have been reported in literature. Certain CT gene families contain multiple members, as well as splice variants and today more than 89 distinct transcripts are known to be encoded by CT genes (Scanlan et al., 2002). A number of CT antigens have been found expressed with high percentage and specificity in HCC. The expression of the CT antigens mRNA was investigated by Wu et al., in the HCC and corresponding peripheral blood of 37 patients with HCC, 15 samples of cirrhotic tissues and 15 normal tissues with the same method. Two CT antigens SSX-2 and SSX-5 showed in this study high specific and high frequent expression only in HCC tissues. In corresponding peripheral blood of HCC tissues, the positive expressions rate of these two CT antigens mRNA was not very high (Benoy et al., 2006). The same group of researchers used another two CT antigens SSX-1 and NY-ESO1 in the same group of patients and with the same methods (RT-PCR) with the corresponding peripheral blood. They showed that SSX-1 can be potential used in peripheral blood, with short term recurrence rate at 46% (6/13) in patients whose peripheral blood expressed SSX-1 mRNA, while the recurrence rate in patients with negative SSX-1 mRNA was 28.6% (4/14) (Bergamaschi et al., 2008). In another study, Peng et al., showed that specific expression of CT antigens was observed in AFP-negative HCC, suggesting the application of their mRNA as tumor markers to detect circulating HCC cells (Peng J. R. et al., 2005). Yang et al., showed that FATE/BJ-HCC-2 (another CTA) mRNA expression was detected in the peripheral blood mononuclear cells (PBMCs) of 46.67% patients, whose HCC tissue samples were cut off and positive for FATE/BJ-HCC-2 mRNA, which implicated tumor cell dissemination in blood circulation and related to the metastasis of HCC. These studies suggest that CT antigens expressions can be used in peripheral blood to detect HCC circulating cells, but

also can be associated to the research of AFP mRNA to increases the specificity and the frequency of the method. This group of markers seems promising and further studies have to be done first to determine the panel of CT antigens to be used as markers of HCC circulating cells.

3.3 New approaches

To attempt the lack of CTC markers, new techniques and technologies are used such as microarray/mRNA large analyses, proteomic and “secretome” analyses and finally microRNA testing.

3.3.1 Microarray/mRNA large analyses

DNA chips were used to measure and find new markers to diagnose HCC, but also to use these as CTC markers. The studies showed the expression of mRNAs for members of the glypican and syndecan families of heparin sulfate proteoglycans such as GPC3 can be a good CTC marker that can be used in human or in mouse models (Suzuki et al., 2010; Yao M. et al., 2011). Another interesting marker was discovered called Snail. Snail mRNA was study in blood of patients with HCC and metastasis (Min et al., 2009). But further investigation has to be done to figure out the specificity and the sensitivity of those markers.

3.3.2 Proteomic and secretome analyzes

In the process to find new markers for CTC, a number of teams started to work with proteomic analysis such as quadrupol IT-TOF, SELDI- TOF MALDI-TOF/TOF mass spectrometry. Their objective is tracking earlier the development and the progression of the HCC. Few markers or group of markers were identified by these methods such as the usual markers AFP, AFP-L3, TGF- β 1, and PIVKA-II but also vitronectin, alpha-1-fucosidase (AFU) and DCP, Golgi protein-73 (GP73), hepatocyte growth factor (HGF), and nervous growth factor (NGF) (Dai et al., 2009; Donati et al., 2010; Liu X. & Wan & et al., 2011; Paradis et al., 2005; Peng X. Q. et al., 2009; Poon et al., 2003; Zinkin et al., 2008).

Interestingly, the proteomic analyses were able to detect new markers in the serum secreted (which is called “secretome”) by the carcinoma cells (Makridakis & Vlahou 2010; Malaguarnera et al., 2010; Niu et al., 2010). Over 90 proteins in some studies compiled with high powerful biocomputational analysis where identified and used to diagnose HCC early (Dai et al., 2009; Paradis et al., 2005; Poon et al., 2003; Zinkin et al., 2008). Unfortunately these studies did not analyze the real usefulness of these markers to identify CTC in the patients with HCC.

A sub-group of HCC was identified by these techniques expressing stem cell markers (CD133, CD90, CD44, EpCAM, CD13 or neural cell adhesion molecule; NCAM) defining what is called now liver cancer stem cell but unfortunately these markers were not studied in the area of circulating tumor cells (Chiba et al., 2009; Huang & Geng 2010; Liu L. L. & Fu & et al., 2011). They are very promising markers. Another group of markers very promising to detect CTC belongs to the chemokine receptors such as CXCR4, CX3CR1 and CCR6 express during HCC progression (Huang & Geng 2010; Li et al., 2010), but none of them were tested during a clinical trial.

3.3.3 MicroRNA markers: A new hope

Around 28 years ago, microRNA (miRNA) was discovered and showed the regulation of genes (Lee et al., 1993; Reinhart et al., 2000) such as oncogenes or tumor suppressor genes at the level of the mRNA. More than 35 studies focused on the identification of miRNA or a group of miRNAs to be used as marker of early diagnosis or metastasis. miR-122/-122a, miR-221/222, miR-145, miR-146a, miR-26 (NF κ B pathway), miR-199a-3p (mTOR pathway) and miR-26 (MYC pathway) were strongly linked to the development and metastasis of the HCC. Also a group of miRNAs were used to identify and classify HCC (Hoshida et al., 2010; Ji & Wang 2009; Kerr et al., 2011; Kojima et al., 2011; Kong G. et al., 2011; Sato et al., 2011), but non of them were used as a CTC marker and tested during a clinical trial.

3.4 Conclusion

In Conclusion, we can observe that not too many specific HCC markers are available and useful for the detection of the CTC. This is certainly due to the heterogeneity of the hepatocellular carcinoma. The most important marker used in clinical routine is the detection of serum AFP mRNA expression (Table 1). But this marker is not expressed in all HCC and by consequence in all CTC leading false negative results. Some propose to combine the research of more than one marker to increase the specificity and the sensitivity of CTC detection method. One of promising marker is Cancer-Testis Antigens but more studies need to be done to select one or more CTA combined (or not) with the detection of the AFP mRNA expression. As we notice previously, CTC are very rare in peripheral blood. We saw also that real-time polymerase chain reaction is a method that in addition to be specific by the nature of the primers used, it can amplify the signal by increasing the number of copies of mRNA originally presents in the sample. But before using RT-PCR, it's necessary to concentrate the number of CTC from the peripheral blood in a smaller volume. The next chapter will describe these methods.

4. Enrichment of the sample

CTC are usually detected in the peripheral circulation, but we can find CTC in other body fluids like the cerebrospinal fluids or the urines. The limitations to discover the CTC in these fluids are the same than in the blood circulation. However, it is possible to extract a relatively big amount of blood without harming the patient and much easier.

We will focus on the methods of CTC detection in the blood. As we describe above, CTC in the peripheral circulation occur at an estimated number of one CTC per 10^{5-7} peripheral blood mononuclear cell or PBMC. Because of the scarcity of the target cells, it is necessary to concentrate the sample. Since enrichment will inevitably be accompanied by some loss of CTC, irrespective of the method, some essays are performed directly in whole blood (Lu Y. et al., 2007).

Two different groups of techniques can be used to enrich samples, the non-specific and the specific enrichment techniques. The non-specific enrichment techniques use physico-chemical CTC properties (size, density, etc). The specific enrichment technique use markers expressed by the CTC. The advantages of the non-specific and specific enrichment techniques are summarized in the Table 2 and described in the following paragraphs.

Methods of Enrichment	Advantages	Disadvantages	References
Non-Specific			
Density (OncoQuick®, Ficoll, UNI-Set®)	<ol style="list-style-type: none"> 1. Isolation of whole and living CTC, 2. Can use another method of enrichment more specific (immunomagnetic beads), 3. Cytopathology, Cytological staining, ICH, FISH...etc can be performed, 4. RNA, DNA extractions followed by RT-PCR or PCR respectively can be done. 	<ol style="list-style-type: none"> 1. Non-specific, 2. Rare CTC can be lost in the plasma fraction or trapped among erythrocyte and neutrophils, 3. Low and variable sensitivity, 4. Depends of the type of CTC, temperature, centrifuge, 5. Expensive. 	(Balic et al., 2005; Becker et al., 2005; Mankin et al., 2002; Paterlini-Brechot & Benali 2007)
Size (MEMS, ISET)	<ol style="list-style-type: none"> 1. Easy 2. Precise counting of the cells per ml of blood, independently of the volume of the blood treated, 3. Allows Cytopathology, Cytological staining, ICH, FISH...etc, 4. Allows Microdissection followed by 5. RNA, DNA extraction follow by RT-PCR or PCR respectively 6. Avoids multiple steps, 7. Increases sensitivity (1 single CTC can be detect from 1ml of blood). 	<ol style="list-style-type: none"> 1. Non-specific, 2. CTC can go through the filter, 3. Cells can be damaged, 4. Expensive, but less than the immunomagnetic beads. 	(Li et al., 2010; Mejean et al., 2000; Paterlini-Brechot & Benali 2007; Pinzani et al., 2006; Vona et al., 2004)
Cytospin	<ol style="list-style-type: none"> 1. Easy, 2. Allows Cytopathology, Cytological staining, ICH, FISH...etc, 3. Allows Microdissection follow by 4. RNA, DNA extraction follow by RT-PCR or PCR respectively. 	<ol style="list-style-type: none"> 1. Increase the mortality of the CTC. 	(Becker et al., 2005; Farina et al., 2004; Kallergi et al., 2008; Kollermann et al., 1999; Saraiva-Romanholo et al., 2003)
Lysis Buffer (Qiagen)	<ol style="list-style-type: none"> 1. Easy, 2. The cells harvested by this method can be re-enriched or analysed by the methods already described, 3. Low cost. 	<ol style="list-style-type: none"> 1. Increase the mortality of the CTC, 2. Low sensitivity. 	(Aryal et al., 2004; Khan et al., 2000; Wharton et al., 1999)

Specific			
ImmunoMagnetic Beads (MACS system, CellSearch)	<ol style="list-style-type: none"> 1. Specific 2. Morphological analysis of CTC, Cytopathology, Cytological staining, ICH, FISH...etc, 3. Multiple labelling of antigens on CTC, 4. Direct quantification of CTC. 	<ol style="list-style-type: none"> 1. Subjective analyses for CTC identification, 2. Time-consuming screening of tumor cells, 3. Need specific marker(s) and antibody available, 4. Expensive. 	(Alix-Panabieres et al., 2005; Alix-Panabieres et al., 2008; Alix-Panabieres et al., 2007; Czerkinsky et al., 1983)
EPI SPOT	<ol style="list-style-type: none"> 1. High sensitivity, 2. Detection of viable CTC, 3. Detection of secreted proteins. 	<ol style="list-style-type: none"> 1. Protein must be actively secreted, shed, or released, 2. No identification and isolation of secreting cell possible. 	(Alix-Panabieres et al., 2005; Alix-Panabieres et al., 2008; Alix-Panabieres et al., 2007; Czerkinsky et al., 1983)
FACS	<ol style="list-style-type: none"> 1. High sensitivity, 2. Technique for counting, examining, and sorting microscopic particles (CTC) suspended, 3. Simultaneous multiparametric analyses of the physical and/or chemical characteristics. 	<ol style="list-style-type: none"> 1. Need the apparatus, 2. High cost. 	(Lobodasch et al., 2007; Mankin et al., 2002; Pachmann et al., 2005)
FAST	<ol style="list-style-type: none"> 1. High sensitivity, 2. Can detect rare events. 	<ol style="list-style-type: none"> 1. Fluorescent dye-conjugated antibodies, 2. Specificity depend of the antibodies, 3. Very expensive. 	(Curry et al., 2004; Hsieh et al., 2006; Lu Y. et al., 2007; Pinzani et al., 2006)
CTC-Chip	<ol style="list-style-type: none"> 1. High sensitivity, 2. Detection of viable CTC. 	<ol style="list-style-type: none"> 1. Detect only cytokeratin+-CTC, 2. Need to control precisely laminar flow conditions, 3. Expensive. 	(Alix-Panabieres et al., 2008; Nagrath et al., 2007)

Table 2. Summary of advantages and disadvantages of the methods of CTC enrichment. CTC, circulating tumor cell; CTC-Chip, circulating tumor cell chip; EPI SPOT, epithelial immunospot; FACS, Fluorescence-activated cell sorting = flow cytometric; FAST, fiber-optic array scanning technology; ICH, immunocytochemistry; ISET, isolation by size of epithelial tumor cells; MACS, magnetic cell sorting; MEMS, micro-electro-mechanical system.

4.1 The non-specific enrichment techniques

4.1.1 Density gradient(s) centrifugation

The tumor cells, epithelial cells, platelets and low density leukocytes from leukocytes and erythrocytes can be separated by the propriety of their particular density (Table 2). Briefly, each cell type has their own density and the assumption of the methods is to put the cells in a buffer with a specific kind of density that usually corresponds to the density of the cells (CTC) that we want to isolate. Density gradient centrifugation is the preferred method to purify cells, subcellular organelles and macromolecules. Density gradients can be generated by placing layer after layer of gradient media such as sucrose in a tube with the heaviest layer at the bottom and the lightest at the top in either a discontinuous or continuous mode. The cell fraction to be separated is placed on top of the layer and centrifuged. Density gradient separation can be classified into two categories: 1). Rate-zonal (size) separation. 2). Isopycnic (density) separation.

Rate-zonal separation takes advantage of particle size and mass instead of particle density for sedimentation. The examples of common applications include separation of cells, cellular organelles such as endosomes or separation of proteins, such as antibodies (Rickwood D; Graham J. M. 2001).

Criteria for successful rate-zonal centrifugation are:

- Density of the sample solution must be less than that of the lowest density portion of the gradient.
- Density of the sample particle (CTC) must be greater than that of the highest density portion of the gradient.
- The pathlength of the gradient must be sufficient for the separation to occur. However, if too long runs are performed, particles may all pellet at the bottom of the tube.

In isopycnic separation, a particle of a particular density will sink during centrifugation until a position is reached where the density of the surrounding solution is exactly the same as the density of the particle. Once this quasi-equilibrium is reached, the length of centrifugation does not have any influence on the migration of the particle. A common example for this method is separation of nucleic acids in a CsCl gradient. A variety of gradient media can be used for isopycnic separations (Rickwood D; Graham J. M. 2001).

Criteria for successful isopycnic separation:

- Density of the sample particle must fall within the limits of the gradient densities.
- Any gradient length is acceptable.
- The run time must be sufficient for the particles to band at their isopycnic point. Excessive run times have no adverse effect (Ford 1991; Graham 1997; Rickwood David. 1992; Rickwood David, Ford T., Steensgaard Jens. 1994; Rickwood D; Graham J. M. 2001).

In the context of the CTC enrichment by centrifugation the isopycnic separation is the method usually used. The cells that have a density higher than the density of the buffer will stay in the bottom of the tube. If the density of the cells is lower than the buffer, they will remain on the top of the liquid, forming a ring. On the contrary, if the density of the cells is the same than the buffer, the cells will form a ring in the middle of the tube. A well known example of the method is the commercial buffer FICOLL™ tube (Amersham

Biosciences AB) or Lymphoprep® (Nicomed) to separate the red blood cells from the other cells including CTC (Table 2).

OncoQuick® (Greiner) method uses a specific buffer able to isolate the CTC (Balic et al., 2005; Mankin et al., 2002; Paterlini-Brechot & Benali 2007). These methods are usually fast but expensive and found in a context of clinical laboratory used in routine diagnosis (Table 2). Alternative and cheaper methods can be used by preparing in the laboratory the gradient/density buffers. The same tube can contain one, two or three gradient buffers to increase the specificity of the separation between the different cells present in the blood (Gertler et al., 2003; Paterlini-Brechot & Benali 2007; Racila et al., 1998; Zach & Lutz 2006).

4.1.2 Lysis buffers

The content of different cells has different osmotic pressures. It is possible to expose the samples to buffer(s) that can be hypo- or hyper osmotic to any cell different to the target cells. After the lysis step the mix is centrifuged and the pellets will contain the CTC. Some companies provide a kit with lysis buffers ready to use. After lysis, the next step is the extraction of DNA or RNA (e.g. Red Lysis Buffer from Qiagen or Panomics) or the extracted cells can be purified by immunomagnetic beads enrichment (Aryal et al., 2004; Khan et al., 2000; Wharton et al., 1999). However, lysis buffer can induce the death of a lot of cells including the CTC and it is not appropriate if the sample contains few CTC leading to false negative results.

4.1.3 Cyto centrifugation (cytospin)

The cyto centrifugation was designed for hypocellular fluids; it spins at lower speeds and has more gradual acceleration and deceleration than normal centrifuges. Some are able to deposit cells directly onto a slide for examination. Cyto centrifugation could be used in research purposes and is also widely used in the routine surgical pathology practice. This method is fast and affordable (Becker et al., 2005; Kallergi et al., 2008; Kollermann et al., 1999; Molnar et al., 2001).

Methods to identify CTC can after be used (see below). As it occurs with magnetic beads, cyto spin increases mortality of the target cells (Table 2). Because enrichment by cyto centrifugation is a critical step, addition of 10 % buffered formaldehyde solution added to the blood sample can preserve morphology of the cells and will certainly preserve nucleic acids integrity (Farina et al., 2004), but the disadvantage of this method is that formaldehyde kills the cells (Table 2). Liquid based cytology (LBC) using a filtration process and computer assisted thin layer deposition of cells has been developed as a replacement for cyto centrifugation and/or smearing, owing to its improved cell recovery capabilities and better cell preservation. In most published series, LBC allows a good interobserver reproducibility. In the urine, processing by the Cytyc ThinprepH 2000 system (Cytyc Corp, Boxborough, Massachusetts, USA) is a method that combines centrifugation and filter transfer methods. The vial containing CTC is placed into a processor (the machine which prepares the smears) together with a glass slide and filter mechanism. The processor immerses the filter assembly into the vial and spins it at a high speed to ensure an even mix of the cells and to break up large cell groups. The fixative is then sucked through a filter membrane which traps the cells but allows fluid through. When an adequate number of

cells have been deposited on the filter, the processor detects a drop in the suction pressure and stops drawing fluid through the filter. It then applies the filter to a specially prepared glass slide and transfers the cells across. The slide is deposited into a vial of fixative (paraffin 10%) from which it is subsequently taken out and stained. It results in increased cellularity and a pronounced reduction of debris, red blood cells (RBC), and crystals (Papillo & Lapen 1994; Piaton et al., 2004; Wright & Halford 2001).

There are several advantages to this system. One is that it produces a thin layer of cells which is easier to evaluate than a thick smear. The morphology of the cells is also better. In addition, the entire cell sample is captured in the fixative vial which leads to a more representative smear being prepared. One of the most important advantages of this test is that the material that is left over after a smear has been prepared can be used for adjunctive testing. A further advantage is that the smears may be initially subjected to image analysis. Computer software “reads” the smears and registers the co-ordinates of the fields with what it regards as the most abnormal cells. On review the system directs the cytotechnologists to these fields where they are evaluated. This can cut down on technologists’ screening time (Table 2).

There are also some disadvantages, which include increased manpower needed to prepare the smears, and the dependence of smear preparation on the instrument. This technique cannot be used directly from blood samples. The red blood cells need to be eliminated by FICOLL® method for example and after the sample can be processed by this technique (Table 2). This method is usually used for urine or ascites samples. However, optimization of cell capture and fixation can be achieved by methods other than Cytoc Thinprep LBC, particularly while using meticulous modern cytocentrifugation methods in the study of hypocellular fluids like in urine for the bladder cancer (Piaton et al., 2004; Wright & Halford 2001). In their study, Piaton et al., conclude that Cytoc Thinprep LBC and modern cytocentrifugation techniques are appropriate methods for cytology based molecular studies. From an economical point of view (standard cytocentrifugation are around \$ 538 compared to Cytoc ThinprepH \$ 1,278), and taking into account the value of a meticulous technique, cytocentrifugation with disposable sample chambers remains the quality standard for current treatment of urinary samples for example (Piaton et al., 2004).

4.1.4 Filters

A non-specific method of enrichment using filters can capture the cells with a certain size. The cells captured on the filter can after be transferred and analyzed on a slide. In this case the samples can come from blood or body fluids (urines, cerebrospinal fluid or ascites). We will describe two kinds of methods using this technology and usually used to isolate CTC (Table 2): the Isolation by Size of Epithelial Tumor Cells (ISET) method and the Micro-Electro-Mechanical System (MEMS).

The Isolation by Size of Epithelial Tumor Cells (ISET) method (Metagenex, Paris, France, www.metagenex.fr) separates cells by size with a filter. Cells larger than 10 μm , including tumor cells from carcinomas, are enriched from leukocytes (erythrocytes are lysed, see above) on a filter. Enriched cells are stained on the filter and CTC are precisely counted after cytopathological evaluation. The cells on the filter can be also studied by immunolabelling,

FISH, TUNEL and molecular analysis. Molecular analysis can be performed specifically to CTC after laser microdissection. The filter can be also mounted between slide and coverslip for routine microscope observation and storage. Although promising, this method is expensive, time consuming and the filters are not easily available (Mejean et al., 2000; Paterlini-Brechot & Benali 2007; Pinzani et al., 2006). Cells should be better characterized using morphological methods that allow both detection and characterization. A second potential main advantage is that CTC could be compared to the primary tumor in order to better understand the mechanism of metastatic process. However, this approach has been rarely performed and neither firm recommendation nor conclusion could be drawn. This method has a high sensibility since one tumoral cell could be detect in 1 ml of blood. The technique also avoids damaging the tumor cell which can be diagnosed using a simple pathologist analysis. However, the pathologist should get used to this technique to avoid a misinterpretation with others types of cells. The use of ISET technology to detect and characterize CTC in HCC has been reported in one study. Vona et al., (Vona et al., 2004) reported that microemboli and isolated CTC could be detected in HCC patient. Presence of CTC was associated with a shorter survival. This work also showed the feasibility of molecular studies of individual circulating cells. Indeed, β -Catenin mutations were searched in samples of 60 single microdissected CTC. β -Catenin mutations were found in only 3 CTC that highlighted the weak impact of these mutations in the initial step of tumor cell invasion. Further studies are needed.

The Micro-Electro-Mechanical System (MEMS)-based is a parylene membrane microfilter device for single stage capture and electrolysis of circulating tumor cells in human blood, and the potential of this device to allow genomic analysis. After the CTC are captured in the filter, electrical lysis of cells on membrane filter is applied and the DNA as well as RNA can be extracted and analyzed by PCR or RT-PCR respectively. CTC enrichment is performed by either gradient centrifugation of CTC based on their buoyant density or magnetic separation of epithelial CTC, both of which are laborious procedures with variable efficiency, and CTC identification is typically done by trained pathologists through visual observation of stained cytokeratin-positive epithelial CTC. These processes may take hours, if not days. The Micro-Electro-Mechanical System (MEMS)-based makes the process simpler, faster and better to separate CTC (~90% recovery) from blood cells. Since enrichment will inevitably be accompanied by loss of CTC, irrespective of the exact method, some essays are performed directly in whole blood (Lu Y. et al., 2007). But the disadvantages of this technique are that morphology of the cells is lost, besides markers also and the capacity to count exactly the number of CTC (Table 2).

4.2 The specific enrichment techniques

The specific enrichment techniques can use specifically protein tumoral markers expressed by the CTC. These methods use antibodies against the protein tumoral markers coupled to steel beads, by applying a magnetic field the cells expressing the marker can be captured. Several immune-magnetic methods (MACS system, Deanabeads® Invitrogen, macro-iron beads, ferrofluid(colloidal iron)-based systems) to enrich the sample have been used successfully (Sergeant et al., 2008). We will describe the methods and their advantages and their disadvantages. Another approach to enrich the sample is to use the properties of CTC

to grow-up in a specific culture cell medium. A method (EPISPOT) that combines the capacity of CTC to secrete specific markers and grow-up in specific cell medium was developed (see Table 2).

4.2.1 Magnetic separation

To use immune-magnetic detection system the first step is to deplete the whole blood of the red cells (by lysis buffers or density gradient) to obtain the PBMC. After, the magnetic particles coated and surrounded by a specific antibody are added to the PBMC supposing containing the CTC. Labeled cells are then collected by applying a magnetic force while non labeled cells are containing in the supernatant and are discerned. This use of magnetic beads to catch specifically CTC is called "positive selection" (Alix-Panabieres et al., 2008; Paterlini-Brechot & Benali 2007; Sleijfer et al., 2007).

Since a large number of leukocytes (potential source of false negative CTC) still remain trapped with the cells, some methods include a "negative selection" of leukocytes (with anti-CD 45 beads for example) followed with a "positive selection" with antibodies specific to epithelial cells (EpCAM, CK) (Allard et al., 2004; Paterlini-Brechot & Benali 2007; Smirnov et al., 2005). The problem of this procedure is that gets rid the majority of leukocytes but still hold in non-malignant epithelial cells and loses tumor cells which do not express epithelial antigens and/or are lysed during the first step (Paterlini-Brechot & Benali 2007; Zigeuner et al., 2003).

The methods using antibodies like immune-magnetic methods (MACS system, Deanabeads® Invitrogen, macro-iron beads, ferrofluid(colloidal iron)-based systems) to enrich the sample will induce false-positive extraction (Sergeant et al., 2008). For example, antibodies against cytokeratin (CK) or other epithelial-specific antigens have been reported to bind both specifically and non-specifically to macrophages, plasma cells and nucleated hematopoietic cells precursors. The non-specific binding of the antibodies involves Fc receptor-bearing leukocytes and monocytes or illegitimate expression of epithelial antigens in normal hematopoietic cells. Some of these positive cells are morphologically difficult to distinguish from CTC. Variable numbers of epithelial cells have been found in peripheral blood of subject without malignancy in some physio-pathological conditions like benign epithelial proliferative diseases, inflammation, surgeries and tissue trauma (Allard et al., 2004; Becker et al., 2005; Goeminne et al., 2000; Naume et al., 2004; Naume & Espevik 1991; Paterlini-Brechot & Benali 2007). Moreover, epithelial CTC may lose epithelial markers during dissemination through the process called epithelial-to-mesenchymal transition (EMT). Since the epithelial markers that get lost during EMT may include markers used for CTC measurement, underestimation of the actual CTC number may occur, inducing de facto false negative results (Christiansen & Rajasekaran 2006; Paterlini-Brechot & Benali 2007; Sleijfer et al., 2007; Wang J. Y. et al., 2006; Willipinski-Stapelfeldt et al., 2005). In the case that the method induce false positive, the problem can be diminished avoided using a second marker or a full panel of markers and techniques (see below) to characterize the CTC, like RT-PCR, immunocytochemistry or immunofluorescence, morphology by optical microscopy (Paterlini-Brechot & Benali 2007; Schuler & Dolken 2006; Sleijfer et al., 2007). In another hand, in the case of the false negative results the doubt persist, and only strict follow-up of the patient by repeating the detection of the CTC can potentially eliminate this doubt. No available antibodies are 100% tumor or tissue-specific (El-Serafi 2004; Goeminne et al., 2000;

Paterlini-Brechot & Benali 2007). To isolate CTC a method using a ligand biotinylated was used. Biotinylated asialofetuin, a ligand of asialoglycoprotein receptor, was experimented and followed by magnetic separation or density gradient (Ficoll-Paque PLUS; GE Healthcare). The cells were identified by microscopy, FISH, immunofluorescence staining, flux cytometry and RT-PCR. This technique shows 81% specificity and 20 cells/5ml for the sensitivity (Xu et al., 2011). This promising approach has to be confirmed in a larger cohort of patients and still depend on the receptors expressed at the surface of the CTC.

4.2.2 Culture of CTC

After isolation of the CTC by the different methods described above, to increase the number of CTC, the primary tumor cells can be cultured in the specific culture medium (Allard et al., 2004). The optimal conditions of culture growth and specially the culture medium leading the growth of the CTC, but not the other epithelial or non epithelial cells, have to be determined through an experiment. Some companies propose commercial kits. For example, The Cancer Cell Isolation Kit® from Panomics includes lysis buffer to increase the number of CTC. One of the main problems is that cultured cells can lose their original markers and derive. Mimicry of tumoral microenvironment *in vitro* is particularly difficult because for most tumors it is largely unknown. Another problem is that the samples containing the CTC are usually contaminated by stromal cells like fibroblasts, which create competition in the Petri dish. After few days, only the fibroblasts are present in the flask.

4.2.3 Epithelial immunoSPOT (EPISPOT)

A technique that allows the detection of only viable cells after a CD45⁺ cell depletion was introduced for CTC analysis from bone marrow aspirates and blood samples (Alix-Panabieres et al., 2008; Alix-Panabieres et al., 2007; Braun et al., 2005). This technique was designated EPISPOT (epithelial ImmunoSPOT). It is a protein-secreting profiling based on the secretion or active release of specific marker proteins using an adaptation of the enzyme-linked immunospot technology. As immunospots are the protein fingerprint left only by the viable releasing epithelial cells, a cell culture is needed to accumulate a sufficient amount of the released marker proteins (Table 2). The dying cells do not secrete adequate amounts of protein and are not detected (Alix-Panabieres et al., 2005; Alix-Panabieres et al., 2008; Alix-Panabieres et al., 2007; Czerkinsky et al., 1983). This assay can also provide important information on the profile of secreted proteins potentially relevant for metastasis formation. However, this technique has still to be validated in large-scale clinical studies on cancer patients (Alix-Panabieres et al., 2008; Alix-Panabieres et al., 2007). After the enrichment and isolation of the CTC, the next step is to identify, characterize and finally enumerate them. The CTC can be identified by indirect or direct methods. But these important steps need tumor markers specific to the CTC sought.

5. Experimental models

5.1 HCC mouse models

Over decades, different HCC mouse models have been developed.

Chemically induced HCC-models are diverse and not always reproducible. The chemicals usually used are diethylnitrosamine (DEN), peroxisome proliferators, aflatoxine, carbon

tetrachloride (CCl₄), choline deficient diet or thiocetamide (Heindryckx et al., 2009; Weylandt et al., 2011). Transgenic mouse models were also developed, for example mice that contained HBV or HCV viruses or expressed specifically oncogenes (c-myc, c-myc + E2F1) or growth factors (TGF- α , TGF- α + c-myc, EGF, FGF19, GMNT, PDGF, α 1-antitrypsin) (Heindryckx et al., 2009). Circulating tumor cells were not looked for in any of these animal models. One reason is the huge differences between models and the presence of specific markers for each situation.

In order to solve these problems, researchers developed ectopic implantation that is fast and easy to perform. However, there is still many differences between the cell lines, no direct interaction with the liver tissues and difficulty to export to humans (Heindryckx et al., 2009).

5.2 Orthotopic implantation

Orthotopic implantation is a more suitable model because the cells are directly implanted in the liver tissue. Nevertheless, the procedure is challenging. There are big differences between cell lines and the choice of the markers is still limited (Heindryckx et al., 2009). Mechanisms leading to tumoral cells spreading are ill known. Currently, there are few models of orthotopic implantation of human tumoral cells (Scatton et al., 2008; Scatton et al., 2006). An experimental model of human orthotopic HCC transplantation in NOD/SCID (non-obese diabetic/several compromise immuno-deficient) mice allows to generate and to modulate CTC (Scatton et al., 2008; Scatton et al., 2006). In this mouse model, tumoral spreading is an early event during tumoral development and the number of CTC is directly correlated to the tumor size.

When injected under the liver capsule, a primary tumor develops and continuously yields circulating tumor cells. In addition, the CTC could be modulated after tumor removal. Liver tumor removal led to a very low level of tumoral cells in blood 30 days later. After complete tumor removal, the number of CTC significantly decreases but still remains detectable even at a low level. The FACS was used to detect CTC (detection of human HLA marker in mouse bloodstream). The reality of CTC was then demonstrated. An important finding is that the bone marrow could be early and permanently colonized by CTC (Scatton et al., 2006).

5.3 Small imaging animal models

With the recent development of the small imaging apparatus (example: IVIS Lumina II XR Imaging System, positron emission tomography) to study development and the progression of diseases in the live animals like rheumatism, a new area to study CTC in live animals is open. This technique was applied to study the CTC in ectopic or orthotopic HCC cell lines implantation. As we discuss above, the lack of specific HCC markers makes CTC studies very challenging. The idea is to bind luminescence tag (luciferase, yellow fluorescence or red fluorescence proteins) in the hepatoma cell lines injected in the liver that be detected by bioluminescence machine. For example, thymidine kinase-luciferase was placed under the transcriptional control of endogenous AFP promoter to develop a transgenic mouse model that injected with DEN will develop HCC (Lu X. et al., 2011). The development of the HCC was followed in the live animal by bioluminescence and PET analyses. The inconvenient of this method is that the HCC model has to express AFP. To avoid this problem hepatoma cell lines were engineered with luciferase (HCC-LM3) (Ma et al., 2011) or red luminescence

protein (Xiao et al., 2011). This approach to study CTC in the context of HCC is very promising, but the major problem is the sensitivity of the bioluminescence machine. This approach has not yet studied in the context of CTC in the blood or in organs other than liver.

6. Conclusion

There are two major problems to detect circulating hepatocarcinoma cells in the human blood. The first problem is the low number of specific markers known. The second problem is that few cells are present in the bloodstream. To overcome these problems, few years ago new approaches have been developed such as the techniques to study membrane proteins by mass spectrometry and the development of fluorescent hepatoma cells.

Nowadays, these procedures are not suitable in clinical practice. However, it is undeniable that early detection of tumors and metastasis is urgently needed in medicine and these new exciting techniques and findings are changing our point of view of carcinogenesis very fast. In the future, CTC detection will certainly be an important diagnostic tool in cancer patients, providing a new and accurate assessment of lesion staging.

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8. References

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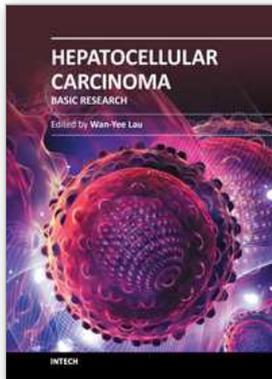
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Hepatocellular Carcinoma represents a leading cause of cancer death and a major health problem in developing countries where hepatitis B infection is prevalent. It has also become increasingly important with the increase in hepatitis C infection in developed countries. Knowledge of hepatocellular carcinoma has progressed rapidly. This book is a compendium of papers written by experts to present the most up-to-date knowledge on hepatocellular carcinoma. This book deals mainly with the basic research aspect of hepatocellular carcinoma. The book is divided into three sections: (I) Biomarkers / Therapeutic Target; (II) Carcinogenesis / Invasion / Metastasis; and (III) Detection / Prevention / Prevalence. There are 18 chapters in this book. This book is an important contribution to the basic research of hepatocellular carcinoma. The intended readers of this book are scientists and clinicians who are interested in research on hepatocellular carcinoma. Epidemiologists, pathologists, hospital administrators and drug manufacturers will also find this book useful.

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