

HEPA-IC

**ELISA Kit for the
detection of
Squamous Cell
Carcinoma Antigen
(SCCA) variants
Immune Complexes
(SCCA-IgM) in
Hepatocellular
Carcinoma (HCC)**

LIFE NANOTECHNOLOGY
XEPTAGEN
LIFE BIOTECHNOLOGY

HEPA-IC

PRODUCT PROFILE

HEPA-IC

code XG003

An Elisa kit designed to assess circulating levels of SCCA-IgM immune complex, a novel HCC biomarker, which may be used for:

Diagnosis of HCC

Hepa-IC may be used for the diagnosis of HCC since the analysis of SCCA-IgM is highly sensitive up to 70% for HCC detection and extremely specific up to 100% in healthy subjects. The assessment of SCCA-IgM has demonstrated higher diagnostic accuracy compared to the reference analysis of AFP levels. Moreover, the high degree of complementarity between SCCA-IgM and AFP levels allows to achieve the best HCC detection using the combination of both tests [1].

Assessment of HCC risk

Hepa-IC may be used to evaluate the risk of HCC evolution in patients with benign liver diseases since the increase of SCCA-IgM levels over time in patients with cirrhosis is prognostic of HCC evolution. SCCA-IgM levels in patients with cirrhosis at high risk of HCC development are higher than those measured in patients at low risk of HCC, thus allowing a serological HCC surveillance based on an annual analysis of SCCA-IgM [2,3].

Assessment of the evolution of liver disease in HCV infected patients

Hepa-IC may be used to monitor the progression of the liver disease in patients infected by hepatitis C virus. The progressive increase over time of serum levels of SCCA-IgM is associated with worsening disease course and histologic deterioration in untreated chronic hepatitis C [4].

Prediction of therapeutic outcome (Theranostic application in HCV treatments)

Hepa-IC may be used to predict the therapeutic outcome of the treatment in HCV infected patients. The antiviral treatment with pegylated interferon and ribavirin induces a significant decrease of circulating SCCA-IgM levels only in patients responding to treatment as measured by a sustained virological response based on the serum HCV-RNA negativity at 24 weeks of follow up [5].

Prognosis of HCC patients

Hepa-IC may be used to determine the prognosis of HCC patients since high levels of SCCA-IgM predict shorter survival. Significantly elevated levels of SCCA-IgM are detected in HCC patients with short-term survival (<36 months) compared to long-term survivors [6].

Monitoring of HCC therapeutic treatments

Hepa-IC may be used to monitor the efficacy of HCC treatments. The decrease of SCCA-IgM levels in patients with locoregional or sorafenib treatment is associated with a positive response to the therapy [7].

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ELISA Kit for the detection of Squamous Cell Carcinoma Antigen (SCCA) variants Immune Complexes in Hepatocellular Carcinoma (HCC)

PRODUCT PROFILE

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Hepatocellular Carcinoma (HCC): from diagnosis to treatment

Hepatocellular Carcinoma (HCC) is one of the most common fatal cancers worldwide, the fourth one for incidence rate. It is the most frequent form of primary liver tumors. Mortality index for this kind of neoplasm is very high: most patients with HCC die within few years after diagnosis, and less than 5% of affected individuals survive to five years (1, 2).

Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) infections, exposure to Aflatoxin B and excessive intake of alcohol have been identified as the major risk factors for HCC development. HBV and, above all, HCV infections are the main causes of chronic liver disease, condition that strongly increases probability of hepatocytes neoplastic transformation. Every year, about 0.5% of chronic liver disease affected individuals develops HCC. This population is defined at high risk of HCC incidence. 300 millions or 170 millions people are HBV or HCV infected all over the world, respectively: this means that about 2,5 millions of HBV or HCV infected persons should be monitored for HCC growth.

Cirrhosis is among the leading causes of death (the third one, precisely) and is also an important risk factor for HCC, irrespective of the etiology of the pathology. The annual risk of developing HCC among persons with cirrhosis is between 1 and 6% and detection of HCC at an early stage is mandatory to improve the poor prognosis of this disease. Risk patients including chronic carriers of hepatitis B and individuals with cirrhosis should be involved in screening programs (3,4).

The clinical outcome of patients with HCC is very poor, since diagnosis is usually established late, treatment is generally unsatisfactory and death often occurs within few years. Except for the presence of α -fetoprotein (AFP) in serum, biochemical tests for the detection of HCC biomarkers are of little help in HCC diagnosis, as at the moment there is no tumor marker specific and sensitive enough to detect HCC in an early phase of development. Sensitivity and specificity of AFP serum level are limited. Only 40-70% of patients with HCC have elevated levels of AFP, whereas only approximately one-third of patients with small HCCs (< 3 cm) have a serum AFP above 200 ng/mL. At a cutoff point of 100 ng/mL, the sensitivity is extremely low. However, specificity and sensitivity is still low to give clinical significance to these assays (Tab.1) (5).

Detection of HCC at an early stage may significantly reduce mortality. This particular cancer develops in more than 90% of cases in patients affected by cirrhosis, a well defined high risk population, and mass screening could be justified since 1) the at-risk population can be easily identified, 2) tumor resection at an early stage can be curative, 3) HCC tends to grow slowly and stay confined to the liver. However massive screening should be justified only when sensitive and specific diagnostic procedures are available. Controlled trials for HCC screening/surveillance in high risk patients have been published and are in progress towards completion. Ultrasonography and α -fetoprotein (AFP) monitoring are at present the only reasonable screening strategies to detect HCC nodules of small dimensions, but not totally satisfactory to justify massive screening programs (6, 7).

"It is therefore important to identify highly specific and sensitive markers in liver tissue, that can predict tumor and tumor staging in an identified at-risk population and in an early stage of development, in order to carry out a timely intervention."

As far as prognosis is concerned, there are many molecular factors (Tab.2), which lately have been considered useful in HCC for therapy response, tumor recurrence and patient

survival monitoring. Proliferation markers, cell cycle/apoptosis regulators, adhesion molecules, angiogenesis promoters are often considered as significant indicators of HCC prognosis, not always with clear results. However, neither one of them nor more put together, at present, provide all the features needed to become a clinical relevant prognostic marker (8).

| METHODS | PROS | CONS |
|--|---|--|
| AFP DETERMINATION | Non invasive (blood samples) | May lead to false positive (> 50 %) and false negative (>30 %) results |
| DCP DETERMINATION | Non invasive (blood samples) | Accurate only for late stage HCC |
| COMPUTERIZED TOMOGRAPHY (CT) SCAN | Non invasive | Accurate when neoplastic nodules are clearly evident (> 3 cm diameter) |
| ULTRASOUNDS (US) | Non invasive | Accurate when neoplastic nodules are clearly evident (> 3 cm diameter) |
| MAGNETIC RESONANCE IMAGING (MRI) | Non invasive | Accurate when neoplastic nodules are clearly evident (> 3 cm diameter) |
| HISTOLOGY (LIVER BIOPSY) | May confirm the diagnosis for lesions \leq 2 cm | Invasive (liver biopsy) Expert pathologist needed |

Tab.1: Comparison among different current methods of HCC diagnosis.

| MARKERS | PROS | CONS |
|---|---|------------------------------------|
| Proliferation markers (PCNA, Ki-67, Mcm2, Mib-1) | Malignant grade evaluation Recurrence time prediction Long-term survival prediction | Low sensitivity Low specificity |
| Nuclear morphology markers (AgNOR) | Tumor stage evaluation Recurrence prediction Progression prediction | Low sensitivity Low specificity |
| p53 and MDM2 | Long-term survival prediction | Low sensitivity Low specificity |
| Cell Cycle regulators (CyclinE, Cdc2, p27) | Recurrence time prediction Long-term survival prediction | Low sensitivity Low specificity |
| Tumor promoters (ras, c-myc, c-erbB-2, EGF-R) | Recurrence time prediction Progression prediction | Low sensitivity Low specificity |

| | | |
|---|---|------------------------------------|
| Apoptosis regulators (Fas, Fas L) | Recurrence time prediction | Low sensitivity Low specificity |
| Adhesion molecules (E-cadherin, ICAM-1, CD44 isoforms) | Tumor stage evaluation | Low sensitivity Low specificity |
| Cancer invasion markers (MMP, uPA) | Recurrence time prediction Long-term survival prediction | Low specificity |
| Angiogenesis promoters (VEGF, bFGF) | Long-term survival prediction | Low sensitivity Low specificity |

Tab.2: Comparison among molecular biomarkers studied for prognostic relevance in HCC.

"It becomes of great importance for HCC control to discover high sensitive and specific prognostic bio-markers."

Therapy of HCC is often just palliative care, based above all on controlling disease and helping patients live longer and better. Localized resectable liver cancer is cancer that can be removed during surgery. Surgical resection provides the best hope but is suitable only in few cases. Patients with small-localized tumors may have prolonged survival after resection, but the diagnosis is usually established late and liver tumor has frequently spread through the liver. Patients with unresectable cancer may receive other treatments to extend life (Tab.3). HCC is not radiosensitive, and chemotherapy is usually unsuccessful. Moderately good long-term survival rates have been reported after liver transplantation (9).

| TREATMENT | METHOD |
|---------------------------------------|---|
| Radiofrequency ablation | Cancer cells necrosis with heat |
| Laser thermal ablation | Cancer cells necrosis with laser-induced heat |
| Percutaneous ethanol injection | Cancer cells necrosis with ultrasound-guided alcohol infusion |
| Hepatic arterial infusion | Cancer cells death with anti-tumoral drug infusion into hepatic artery |
| Chemoembolization | Cancer cells death with anti-tumoral drug infusion into hepatic artery, followed by blocking the flow of blood through the artery |

Tab.3: Treatment choices other than surgery in HCC therapy. At this time, liver cancer can be cured only when it is found at an early stage and only if the patient is healthy enough to have an operation.

Serological Tumor Markers for HCC Diagnosis

The most widely used serologic marker to detect HCC is α -fetoprotein (AFP), which is elevated (>20 ng/mL) in a wide number of HCC patients (30-60%) but with low specificity (70-80%) since a considerable number of patients with chronic liver disease may have AFP levels in the range 20-200 ng/mL (10-12). In addition, AFP serum levels in cirrhotic and HCC patients often overlap and higher AFP cut off values (>100 ng/mL) have been used to increase specificity but reducing sensitivity to extremely low values (5-15%) (13). Given the high heterogeneity of HCC (14), other biomarkers have been found to be overexpressed in the liver and/or serum of patients.

Des- γ -carboxy prothrombin levels have been found elevated in 35-53% of HCC patients (15-16) as a result of an acquired defect in the post translational carboxylation of the prothrombin precursor in neoplastic cells (17). While some studies reported usefulness of this marker compared to AFP (18), others found no improvement over AFP determination recommending a combination of both assays to improve sensitivity and specificity (19).

Glypican-3 (GPC3) messenger RNA levels have been found to be overexpressed in the liver in 75% of HCCs but in only 3.2% of normal livers (20). Immunohistochemistry results confirmed the occurrence of GPC3 protein in 72% of cases (21) and by ELISA circulating GPC3 protein has been detected in 40-53% of the patients with HCC (21-22). Other markers proposed for HCC surveillance, including lectin-reactive AFP, p53 autoantibodies, carbohydrate-deficient-transferrin, hepatitis B virus (HBV) encoded X antigen, and alpha-L-fucosidase, lack adequate specificity to support a diagnostic value (23).

Overexpression of squamous cell carcinoma antigen (SCCA) variants (SCCA-1, SCCA-2 and SCCA-PD) has been recently reported in all surgically resected HCC but in none of the control normal livers, as detected by immunohistochemistry (24). SCCA is a serine protease inhibitor physiologically found in the spinous and granular layers of normal squamous epithelium, but typically expressed by neoplastic cells of epithelial origin (25). Both SCCA isoforms SCCA1 and SCCA2 (26) protect neoplastic cells from apoptotic death induced by several kinds of stimuli and in vivo experiments demonstrate that SCCA1 can promote tumor growth (27-28).

Hepa-IC ELISA Kit

ELISA Kit for the detection of Squamous Cell Carcinoma Antigen (SCCA) variants Immune Complexes associated to Hepatocellular Carcinoma (HCC)

Early detection of HCC is still difficult due to the lack of adequate biomarkers to clearly differentiate HCC from benign liver lesion with high sensitivity and high specificity. A new biomarker for HCC, Squamous Cell Carcinoma Antigen (SCCA) variants, has been recently identified in all surgically resected HCC but in none of the control normal livers, as detected by immunohistochemistry with anti-SCCA variants antibody (Hepa-Ab, XEPTAGEN) (24). In HCC patients sera, SCCA variants are detected as circulating immune complexes SCCA-IgM, with 70% sensitivity and 100% specificity versus healthy subjects. XEPTAGEN has developed Hepa-IC, an ELISA kit for the assessment of SCCA variants as circulating immune complexes (SCCA-IgM) in patients sera. Hepa-IC is highly specific and sensitive for HCC detection. The amount of SCCA-IgM was expressed in Arbitrary Units (AU/mL), using a reference standard curve.

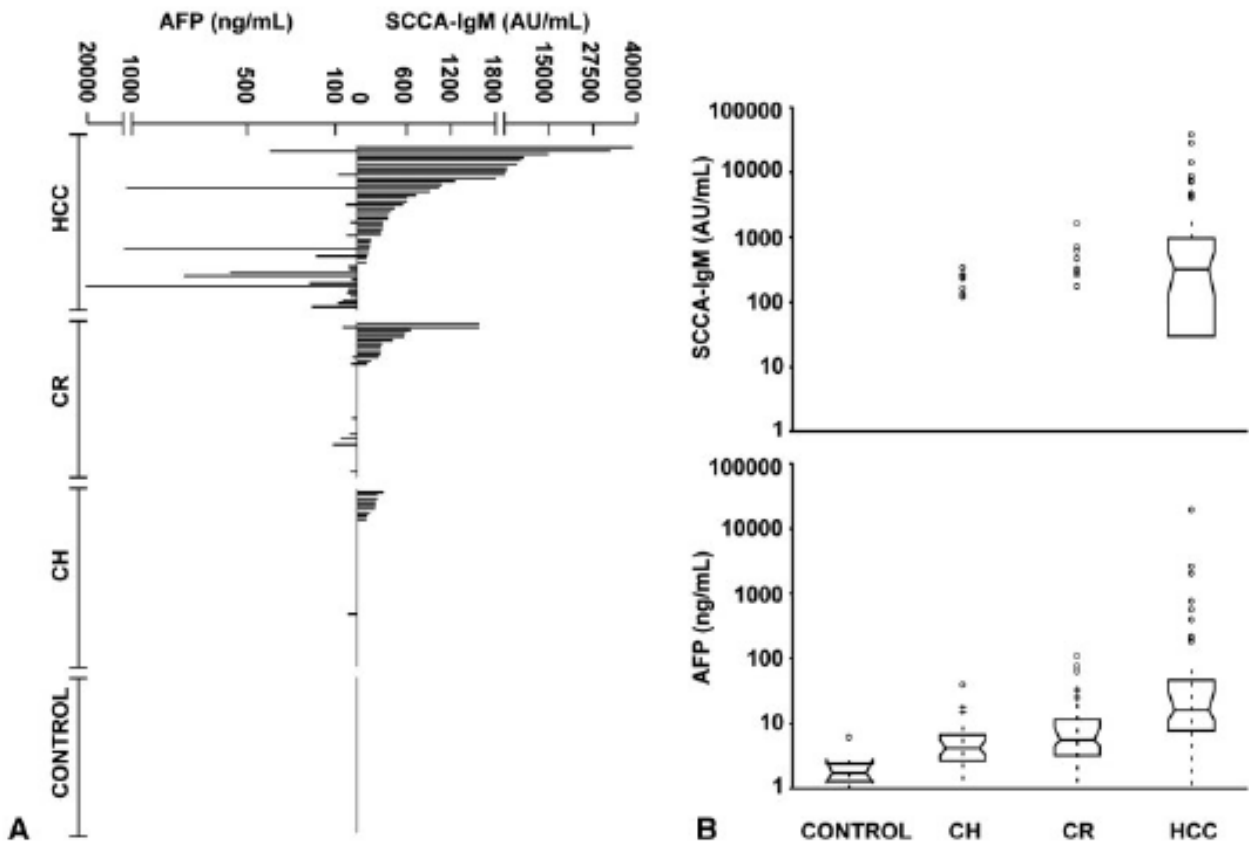


Fig. 1: A: Significantly elevated serum levels of SCCA-IgM and AFP in HCC cirrhosis chronic hepatitis and in controls and as detected by ELISA. **B:** Box plot for SCCA-IgM (top) and AFP (bottom) values in the four groups of subjects. The box indicates the lower and upper quartile and the middle line indicates the median. Boxes are notched at the median with the lengths of the notches representing the 95% confidence interval. A dotted-line connects the observations within 1.5 inter-quartile ranges (IQRs) of the lower and upper quartile. Crosses represent the observations between 1.5 and 3.0 IQRs from the quartiles and circles represent points beyond this.

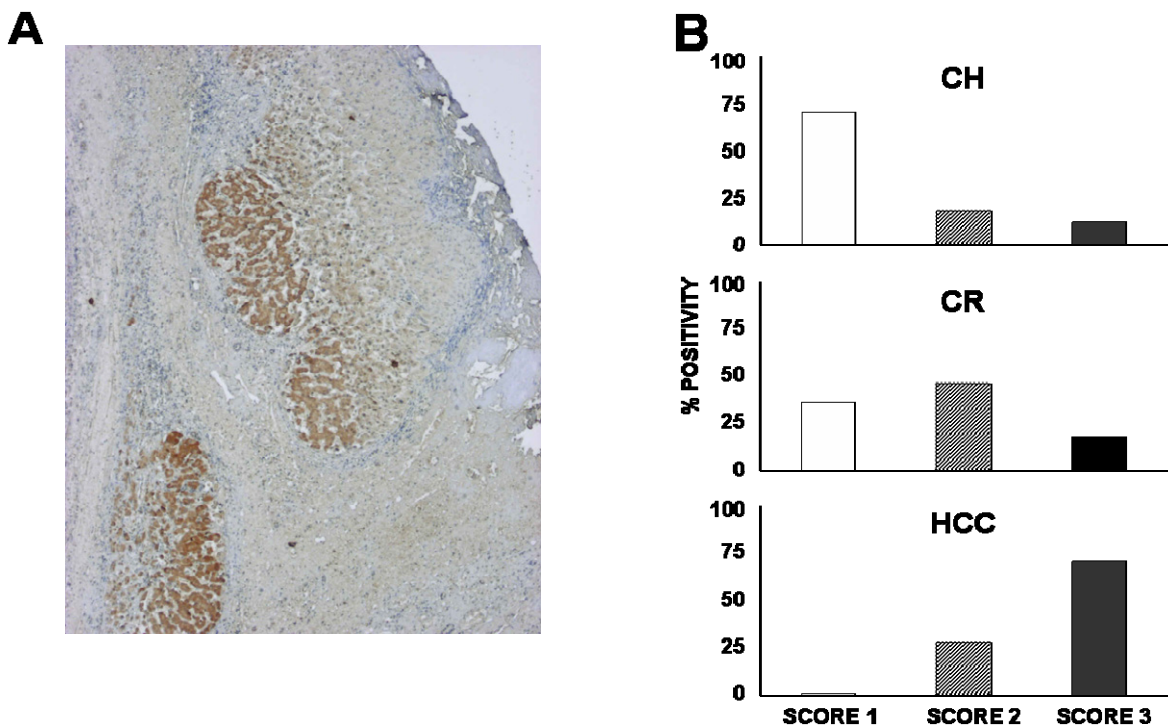


Fig.2: A. SCCA expression in cirrhotic liver. **B.** SCCA score distribution in liver specimens showing SCCA reactivity in the different groups of patients.

The usefulness of circulating SCCA-IgM in terms of sensitivity and specificity for HCC detection was determined and compared to AFP measurement. Serum samples from 160 patients with different spectra of liver disease but no concomitant diseases and from 73 healthy donors were analyzed (29, 30). Patients with liver disease included 60 cases with HCC (age mean \pm SD = 64 \pm 14 years, M/F ratio = 2:1) and the etiology was HCV in 80%, HBV in 18%, and both HBV/HCV in 2% of the cases. A group of 50 patients with cirrhosis (age mean \pm SD = 51 \pm 9 years, M/F ratio = 2:1) was also included and they were positive for HCV in 86% of the cases and for HBV in 14%. The third group included 50 patients with chronic hepatitis C (age mean \pm SD = 44 \pm 12 years, ALT level mean \pm SD = 96.19 \pm 81.06 U/L, M/F ratio = 1:1). Preliminary investigations by ELISA on sera from HCC patients indicated that free SCCA was detectable with low sensitivity and low specificity when compared to healthy subjects (data not shown). The situation was remarkably different when SCCA Immune Complexes (SCCA-IgM) were evaluated.

By using Hepa-IC the vast majority of HCC samples (70%) are strongly reactive (mean \pm SD = 2568.5 \pm 6797.3 AU/mL), while all healthy controls (n=73) are negative (<120 AU/mL) (Fig 1A). In cirrhotic patients SCCA-IgM immune complexes are detected in 26% of cases but at lower levels (mean \pm SD = 147.5 \pm 348.3 AU/mL). Patients with chronic hepatitis C in only 18% of cases display presence of SCCA-IgM but at very low levels (mean \pm SD = 39.5 \pm 89.7 AU/mL) (Fig 1B). The same samples were tested in parallel for AFP content, but no correlation was found with AFP levels, which were significantly elevated (> 20 ng/mL) only in 42% of HCC patients (29, 30, 31). By using an AFP cut off value of 20 or 100 ng/mL, 96 or 80% respectively of HCC patients were positive for at least one marker.

Concentration of circulating SCCA-IgM paralleled the extent of SCCA overexpression detected by immunohistochemistry in liver specimens (Hepa-Ab, XEPTAGEN). In patients with chronic hepatitis SCCA was detectable in the liver of 50% of the cases, showing 65% of the reactive samples with score 1 (< 30% positive hepatocytes). The percentage of SCCA reactivity increased to 75% in patient with cirrhosis, where 50% of livers specimens stained with score 2 (30-50% positive hepatocytes), while in HCC 93% of the samples were reactive and 70% of them presented score 3 (> 50% positive hepatocytes) (Fig. 2) (29, 30, 31)

Table 4 shows a comparison of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) between SCCA-IgM and AFP levels (20 ng/mL cut-off) in patients with liver pathologies and normal control (29,30).

Increase of SCCA-IgM Complexes and HCC Development in Cirrhotic Patients

Work by Pontisso et al. demonstrated that in cirrhotic patients the progressive increase of SCCA-IgM over time was significantly associated to tumor progression, rather than the absolute concentration of the immune complex (32, 33).

A retrospective, longitudinal study was conducted in a group of 16 cirrhotic patients (group A) who developed HCC during a median follow up of 4 years (range 2-8 years) and in a group including 17 control patients with cirrhosis, who did not develop HCC during the same time interval (group B).

Both groups had similar clinical and epidemiologic profile at presentation and SCCA-IgM complexes reactivity did not significantly differ in two groups, [mean \pm SD: 267.40 \pm 382.25 AU/mL vs 249.10 \pm 446.90 AU/mL, p = 0.9006]. Alpha-fetoprotein did not correlate with the presence of SCCA-IgM in the same serum sample (r = -0.11), being AFP values similar in both groups. The increase over time of SCCA-IgM (Φ) was significantly higher in cirrhotic patients who developed HCC compared to those who did not progressed to liver cancer (Φ mean \pm SD = 280.05 \pm 606.71 (AU/mL)/year vs. -37.92+95.94 (AU/mL)/year, p=0.0408). (Fig. 3)

While in the majority of patients of group A the increase over time of SCCA-IgM was >20 (AU/ml)/year, the same behaviour was observed only in 6% of the patients of group B, where no increase or a slight decrease of Φ were observed in 76% of the cases (Fig. 4).

In conclusion, the monitoring of SCCA-IgM levels over time in cirrhotic patients appears a useful parameter to predict HCC development, allowing special focusing of therapeutic strategies with increased velocity.

The clinical use of SCCA-IgM assay for monitoring patients with cirrhosis

A multicenter retrospective longitudinal study on 56 patients with HCV-related cirrhosis monitored for 12-72 months has demonstrated that the use of Hepa-IC kit may reduce the need of ultrasound surveillance of patients with cirrhosis without interferon treatment.

The results of the study have proved the high diagnostic accuracy of SCCA-IgM test in the detection of patients with cirrhosis at lower risk of developing HCC demonstrating that 28 patients with HCV-related cirrhosis who did not develop HCC after at least 12 months of follow-up displayed SCCA-IgM levels persistently below 300 AU/mL (Table 5). On the other hand patients with HCV-related cirrhosis at higher risk of HCC

| BIOMARKER | Sensitivity | Specificity | PPV | NPV |
|---|-------------|-------------|------------|-----------|
| SCCA-IgM 120 AU/mL | | | | |
| HCC vs Control | | 100 | 100 | 77 |
| HCC vs CR | 70 | 74 | 73 | 71 |
| HCC vs CH | | 82 | 80 | 73 |
| AFP 20 ng/mL | | | | |
| HCC vs Control | | 100 | 100 | 63 |
| HCC vs CR | 42 | 84 | 72 | 59 |
| HCC vs CH | | 98 | 95 | 63 |
| SCCA-IgM 120 AU/mL & AFP 20 ng/mL | | | | |
| HCC vs Control | | 100 | 100 | 96 |
| HCC vs CR | 96 | 64 | 73 | 94 |
| HCC vs CH | | 80 | 83 | 95 |
| SCCA-IgM 120 AU/mL & AFP 100 ng/mL | | | | |
| HCC vs Control | | 100 | 100 | 83 |
| HCC vs CR | 80 | 72 | 74 | 78 |
| HCC vs CH | | 82 | 82 | 80 |

Tab. 4: Comparison of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of SCCA-IgM and AFP, in differentiation of patients with HCC (n=50) from those with cirrhosis (CR, n=50), chronic hepatitis (CH, n=50) and healthy subjects (n=50 controls).

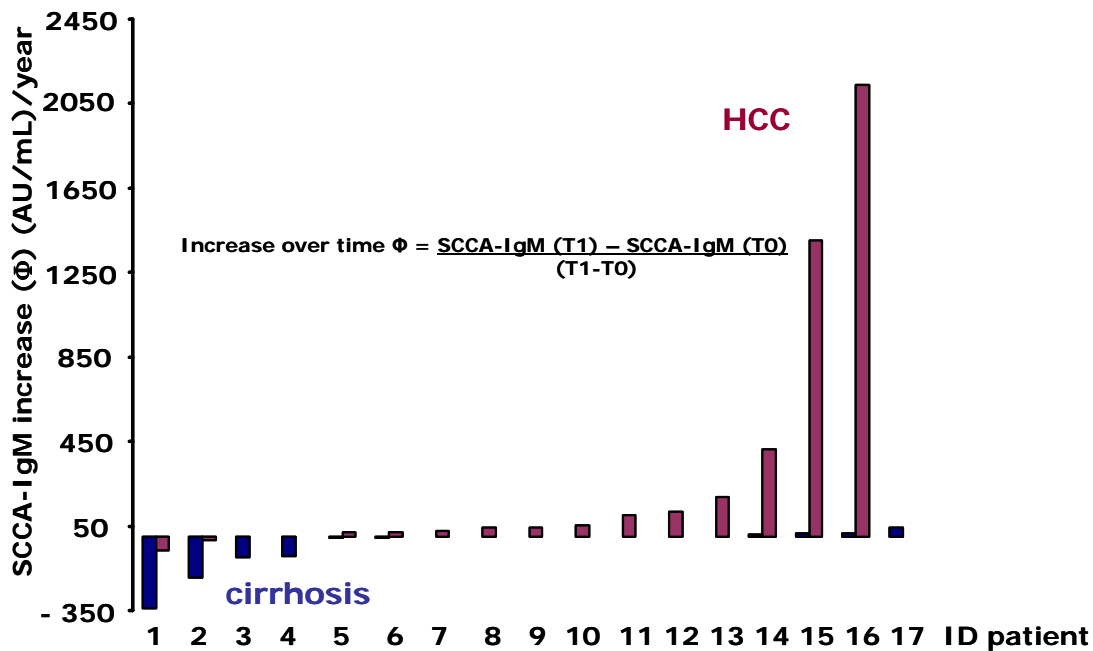


Fig. 3: Determination of the increase over time (Φ) of SCCA-IgM for each component of the group of cirrhotic patients who developed HCC during follow up (HCC) and in the group of cirrhotic patients who did not develop HCC during the same interval of observation (cirrhosis)

SCCA-IgM IC (Φ score) in cirrhosis and in HCC

- $\Phi 1 \Rightarrow$ SCCA-IgM IC ≤ 0 AU/mL
- $\Phi 2 \Rightarrow 0 < \text{SCCA-IgM IC} \leq 20$ AU/mL
- $\Phi 3 \Rightarrow$ SCCA-IgM IC > 20 AU/mL

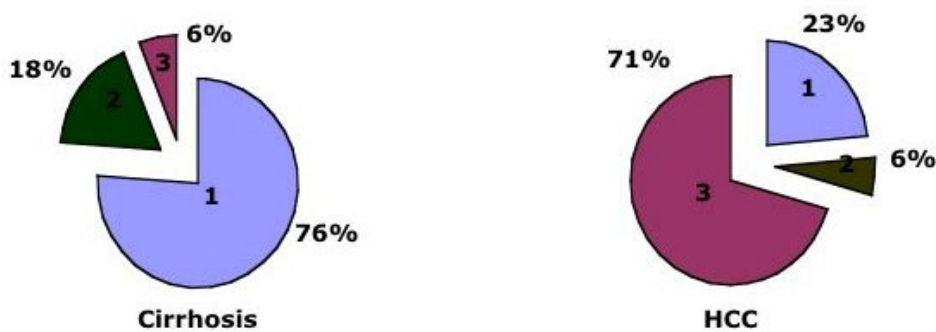


Fig. 4: SCCA-IgM IC (Φ score) in cirrhosis and in HCC patients. They grouped on the basis of different values of increase of SCCA-IgM (AU/mL)/year.

development showed SCCA-IgM levels above 525 AU/mL. (Table 6).

The annual assessment of circulating levels of SCCA-IgM could allow the identification of patients at lower risk of HCC development (SCCA-IgM < 300 AU/mL) for which the surveillance for an early diagnosis of HCC based on liver ultrasonography is not cost-effective since they have a trifling risk of developing an HCC at mid-term. Eliminating the ultrasound surveillance in these patients would permit to improve significantly the cost/effectiveness of the surveillance

for an early HCC diagnosis presently based solely on the ultrasound technique, which is currently due for all patients with cirrhosis into Child Pugh class A or B (34).

Based on the study results a model for the clinical application of Hepa-IC kit (Figure 5) for monitoring patients with cirrhosis has been proposed. The model assumes a serological surveillance with an annual analysis of SCCA-IgM levels in patients with cirrhosis which are negative at the first US analysis.

| SCCA-IgM Cut off (AU/mL) | Prevalence (%) | Se (%) | Sp (%) | PPV (%) | NPV (%) |
|--------------------------|----------------|--------|--------|---------|-------------|
| 300 | 50 | 57.1 | 89.3 | 84.2 | 67.6 |
| | 5 | 57.1 | 89.3 | 21.4 | 97.5 |

Tab 5: Indexes of sensitivity (SE), specificity (SP), positive and negative predictive values (PPV,NPV) for SCCA-IgM assay in 56 patients with HCV-related cirrhosis monitored for 12-72 months before HCC diagnosis or the end point of the study and in a simulation assuming a prevalence of 5% as the annual incidence of HCC. The cut off of 300 AU/mL allows the identification of patients at lower risk of HCC (NPV=97,5%).

| SCCA-IgM Cut off (AU/mL) | Prevalence (%) | Se (%) | Sp (%) | PPV (%) | NPV (%) |
|--------------------------|----------------|--------|--------|------------|---------|
| 525 | 50 | 42.9 | 100 | 100 | 63.6 |
| | 5 | 42.9 | 100 | 100 | 97.1 |

Tab 6: Indexes of sensitivity (SE), specificity (SP), positive and negative predictive values (PPV,NPV) for SCCA-IgM assay in 56 patients with HCV-related cirrhosis monitored for 12-72 months before HCC diagnosis or the end point of the study and in a simulation assuming a prevalence of 5% as the annual incidence of HCC. The cut off of 525 AU/mL allows the identification of patients at higher risk of HCC (PPV=100%).

Monitoring SCCA-IgM Complexes in Serum Predicts Livers Disease Progression in Patients With Chronic Hepatitis

About 30% of the patients with chronic hepatitis develop a progressive liver disease and one of the most intriguing issues is the detection of non invasive markers for fibrosis stage and disease progression. High levels of SCCA-IgM are detectable in hepatocellular carcinoma and their increase in cirrhotic patients can predict tumor development. Since SCCA-IgM can also be detectable in low percentage in patients with chronic hepatitis, a study was conducted to assess SCCA-IgM complexes in relation to disease outcome in this group of patients (35).

In this study SCCA-IgM complexes were determined by Hepa-IC in 188 patients with chronic hepatitis and in 100 controls. In 57 untreated patients an additional serum sample was available after a median period of 6 years: these patients were divided in group A, including 8 patients with fibrosis score increase >2 in the second liver biopsy and group B, including 49 patients without fibrosis progression during similar follow up. At presentation circulating SCCA-IgM immune complexes were detectable in 63/188 (33%) patients with chronic hepatitis, but in none of the control group. No different frequency of reactivity was observed in chronic patients with HBV or HCV infection, being reactive 27.5% of the HBV positive patients and 34% of those with HCV infection. Mean age (47 ± 13 years vs 42 ± 14 years, $p= 0.05$) and sex distribution of the reactivity (M/F: 0.52 vs 0.48 $p= 0.457$) were similar in patients with and without SCCA-IgM complex.

In patients followed over time, a higher percentage of SCCA-IgM reactivity at presentation was detected in the group of patients

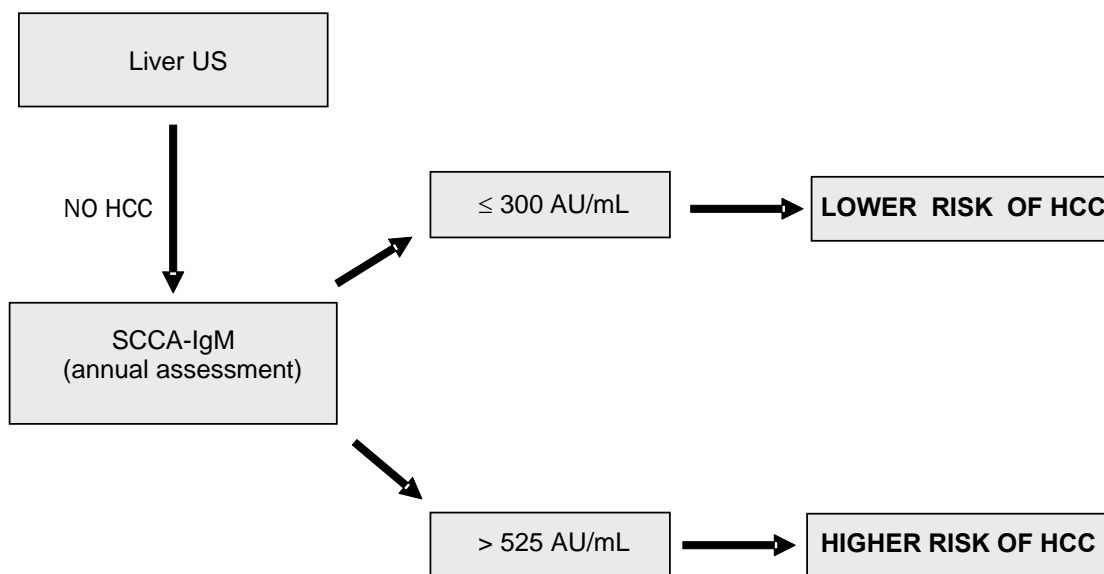


Fig 5: Model of serological surveillance with an annual analysis of SCCA-IgM levels in patients with cirrhosis which are negative at the first US analysis

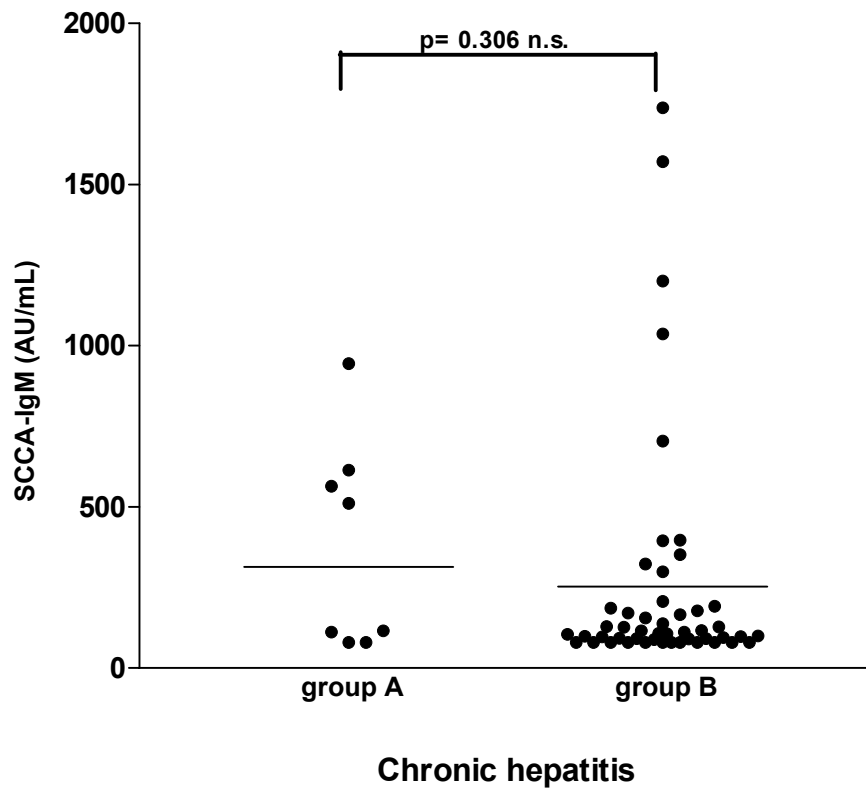


Fig. 6: Distribution of serum level of SCCA-IgM at presentation in patients with chronic hepatitis with (group A) or without (group B) disease progression over time. Horizontal bars represent median values in the two groups. Figure 6 depicts median values of SCCA-IgM complex detected at presentation (T1) and in the second time point (T2) in the two groups of patients with different disease evolution. Levels of the complex were substantially stable over time in patients without disease progression (median value T1=108 AU/ml T2= 94 AU/ml), while an increase was detected in 75% of the patients with progressive disease (median value T1= 313 AU/ml, T2= 707 AU/ml, $p= 0.014$)

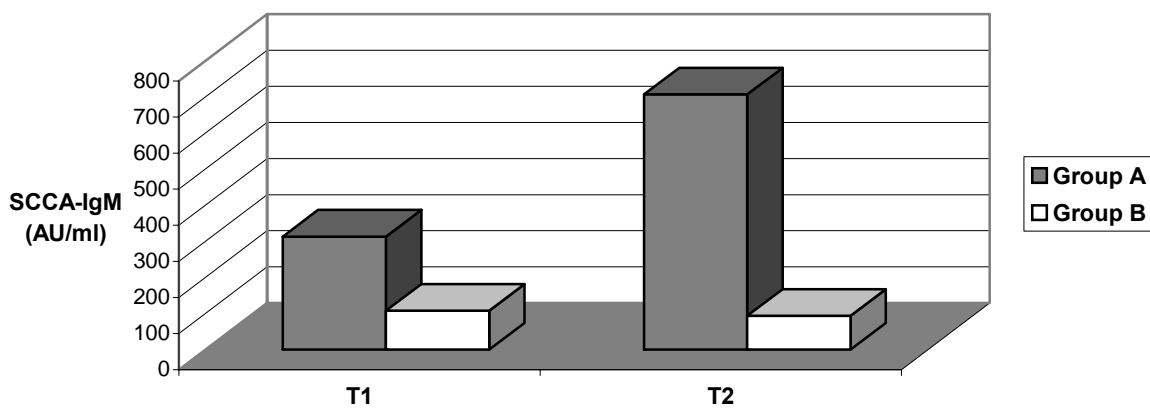


Fig. 7: SCCA-IgM median values at first (T1) and at second (T2) serum test time in patients with chronic hepatitis in the two groups of patients with chronic hepatitis and with disease progression (group A) or without disease progression (group B).

with subsequent disease progression (group A), compared to patients without histologic evolution (group B), although not reaching statistical significance (50% vs 33%, $p= 0.432$ n.s.). Serum levels of SCCA-IgM in reactive cases, however, were not different in the two groups (mean \pm SD, group A: 378 ± 326 AU/ml; group B: 252 ± 369 AU/ml, $p=0.306$), as shown in Fig 6. To better analyse these results, the increase of SCCA-IgM over time (Φ) was calculated for each patient. A significant increase of SCCA-IgM during follow up was detected in patients with chronic hepatitis and progressive disease, but not in those

without histologic progression (Φ mean \pm SD: 117 ± 200 U/year vs -8.8 ± 31 U/year, $p < 0.0001$). As displayed in Figure 8, the distribution of Φ values in this latter group of patient, mainly characterized by negative values, reflects a decrease of this parameter over time in the majority of the patients without disease progression. Authors conclude that monitoring SCCA-IgM complex behaviour over time could become an useful approach to predict disease outcome in individual patients with chronic hepatitis.

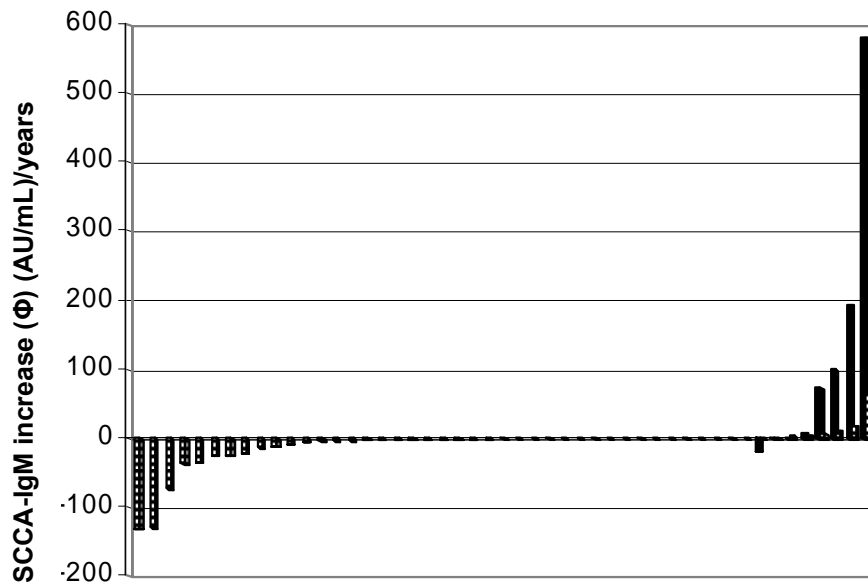


Fig. 8: Distribution of the increase of SCCA-IgM over time (Φ) in individual patients. Full bars refer to patients with liver disease progression (group A) and dashed bars refer to patients without disease progression (group B).

Prediction of therapeutic outcome of antiviral treatment

A successful antiviral therapy with pegylated interferon (PEG-IFN) and ribavirin may decrease the incidence of HCC in patients with HCV related cirrhosis stopping the disease progression (36). The well known immunomodulatory effects of pegylated interferon (PEG-IFN) antiviral therapy have led to evaluate the behaviour of circulating levels of SCCA-IgM immune complexes for monitoring the success rate of PEG-IFN and ribavirin treatment. Serum samples from a cohort of 33 patients chronically infected with HCV and clinically or histologically proven compensated cirrhosis who underwent combined PEG-IFN and ribavirin antiviral therapy were analyzed for SCCA-IgM titer by Hepa-IC.

Treatment duration with PEG-IFN and ribavirin was 24-48 weeks according to viral genotype. Sustained virological response (SVR) was obtained in 15 patients on the basis of serum HCV-RNA negativity at 24 weeks of follow-up even if treatment was discontinued before as a result of side effects or non-compliance to therapy. Nonresponse (NR) to treatment was defined as lack of HCV-RNA clearance during treatment and at follow-up.

The SCCA-IgM assessment was performed at baseline, at the end of treatment, at 6-month and 12-month follow-up.

Serum levels of SCCA-IgM decreased significantly over time from 59% at the end of treatment (median level of SCCA-IgM= 186.8 AU/mL) to 89% in SVR patients at 12-month follow up of antiviral therapy (median value of SCCA-IgM= 52.4 AU/mL) compared to baseline (median level of SCCA-IgM= 451.2 AU/mL, Figure 9) whereas NR patients had stable levels of SCCA-IgM in the bloodstream at the end of treatment and during follow-up (37).

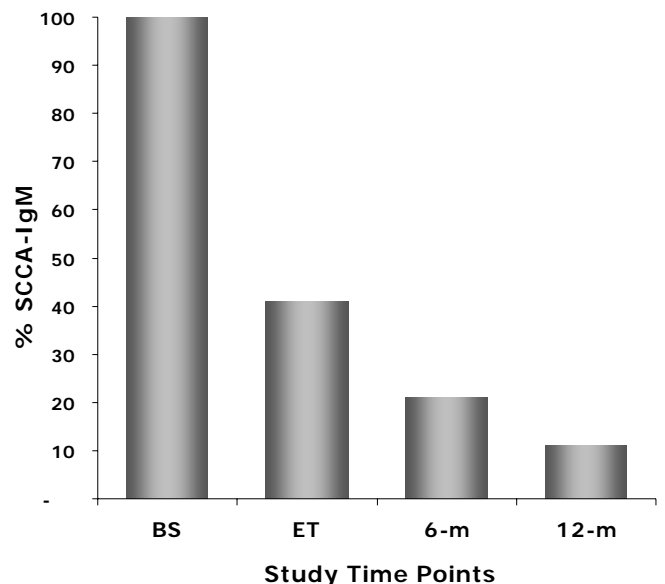


Fig. 9: Percentage of serum levels of SCCA-IgM in patients with HCV related cirrhosis at baseline (BS), at the end of treatment (ET), at 6-month (6-m) and 12-month (12-m) follow up in patients with SVR after antiviral therapy.

In summary, SCCA-IgM may be used in the clinical setting to predict the therapeutic outcome of the antiviral treatment with PEG-IFN and ribavirin in cirrhotic patients with HCV infection.

Monitoring of HCC therapeutic treatments

A prospective study aimed to evaluate the ability of SCCA-IgM levels of predicting the efficacy of therapy in different HCC treatments was performed in two referral clinical centres (38). Sixty patients with a new diagnosis of HCC were enrolled and divided into two groups according to the therapy received. Thirty-five patients of the first group were treated with locoregional therapy (RF and Laser ablation, PEI, TACE) while twenty-five patients of the second group received the treatment with sorafenib. For clinical management of HCC the Barcelona-Clinic Liver Cancer (BCLC) classification was used. Measurements of serum SCCA-IgM levels were made at the basal time and after 1 and 3 months from the beginning of the treatment. The response to the therapy was evaluated with imaging techniques according to mRECIST criteria.

At the basal time, 19 out of 35 patients in Group 1 were positive for SCCA-IgM (mean value \pm SD, 305 \pm 344 AU/mL, whereas 11 out of 25 patients in Group 2 were reactive for SCCA-IgM (mean value \pm SD, 353 \pm 280 AU/mL). Among the 19 positive patients in Group 1 a reduction of serum SCCA-IgM was determined in 79% (15/19) of cases during follow up after locoregional treatments and a similar decrease was observed in 64% (7/11) of positive patients in Group 2 during therapy with sorafenib. Furthermore, 86% (19/22) of total patients in both groups displaying decreased SCCA-IgM levels obtained a positive response to locoregional or Sorafenib therapy (38). In conclusion, the assessment of SCCA-IgM levels in patients undergoing HCC treatments may be helpful in monitoring the outcome of the therapy (38).

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Hepa-IC - Product Data Sheet

INTENDED USE

Hepa-IC is an enzyme linked immunosorbent assay (ELISA) for the quantitative measurement of Squamous Cell Carcinoma Antigen (SCCA) variants immune complexes (SCCA-IgM).

SUMMARY AND EXPLANATION OF THE TEST

Hepa-IC is an innovative in-vitro diagnostic method based on the detection of SCCA variants as circulating Immune Complexes (IC). Hepa-IC is a highly specific and sensitive ELISA assay for HCC detection designed to measure SCCA-IgM in patients sera. The amount of SCCA-IgM is expressed in Arbitrary Units (AU), using a specific calibrator as reference. The measurement of SCCA-IgM offers the possibility to remarkably increase HCC detection sensitivity without compromising specificity compared to the serum levels of α -fetoprotein (AFP) (1,8,9,11-15). The assessment of SCCA-IgM has also been found to be useful in the monitoring of HCC development in chronic hepatitis (CH) and cirrhotic (CR) patients (2-7,10).

PRINCIPLE OF THE TEST

Standard Calibrators and specimens are simultaneously incubated with anti-SCCA variant antibodies coated to the wells of a microtiter plate. The immune complexes SCCA-IgM is detected by the addition of an enzyme conjugated secondary antibody and an enzyme substrate (ABTS). The developed color is proportional to the amount of the analyte in the sample.

REAGENTS AND MATERIALS PROVIDED

XG003-PL: 96 wells multi-strip Assay-Plate, pre-coated with affinity purified rabbit anti-SCCA

XG003-Calibrator: Two vials of calibrator lyophilized from PBS. White powder. Exact concentration on label. Totally soluble.

XG-EA: 1.1 mL of Enzyme-conjugated goat anti-human IgM secondary antibody (Green cap) 10-fold concentrate solution in PBS containing 1% BSA.

XG-CH4: Chromogen: ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic-acid)); Buffer: phosphate citrate. Light green tablet + white powder lyophilized from 0.05 M phosphate-citrate buffer, pH 5. Totally soluble.

XG-SB: 200 μ L of Enzyme substrate: hydrogen peroxide solution 30% (w/w) in water. (Blue cap)

XG-DB5: Concentrated Dilution Buffer solution 5X, 10 mL. Once diluted, the working solution contains 1% BSA and 0.05% Tween 20 in PBS. The solution contain Proclin as preservative.

XG-WB2: Lyophilized Washing Buffer. Two white tablet. Once diluted, the working solution contains 0.05% Tween 20 in PBS. Totally soluble.

MATERIAL AND EQUIPMENT REQUIRED

Precision pipettes with disposable tips

Microplate washer

Microplate readers with a 405 ± 20 nm filter

Distilled or deionized water

STORAGE CONDITIONS

Storage at 4 °C:

XG003-PL, XG-CH4[†], XG-SB, XG-DB5^{*}, XG-WB2^{*}

Storage at -20 °C:

XG-EA, XG003-Calibrator[§].

Avoid repeated freeze and thaw cycles

(*) Must be used within one month of reconstitution

(§) Must be reconstituted just before the use

(†) Must be stored in a dark location

EXPIRATION DATE

Expiration date printed on the kit indicates limits of stability.

WARNINGS - POTENTIAL BIOHAZARDOUS MATERIALS

The XG003-Calibrator contains proteins of **human origin**. The reference material was tested using an approved method of evaluation for the presence of the antibodies to HIV, antibodies to the hepatitis C virus and hepatitis B surface antigens, and found to be negative. **Since no test method can offer complete assurance that HIV, hepatitis B virus, hepatitis C virus, or other infectious agents are absent, all human sourced materials should be considered potentially infectious.** It is recommended that these reagents and human specimens be handled in accordance with the OSHA Standard on Bloodborne Pathogens (16). Biosafety Level 2 (17) or other appropriate biosafety practices (18, 19) should be used for materials that contain or are suspected of containing infectious agents.

SPECIMEN COLLECTION AND PREPARATION

The use of serum samples are recommended for the Hepa-IC assay.

Serum specimens should be collected aseptically, avoiding hemolysis if possible.

Specimens should be stored at 2-8 °C if the assay will be performed within 24 hours after collection. Specimens should be stored frozen if testing will occur after 24 hours.

If frozen, specimens should be mixed thoroughly after thawing to ensure consistency in the results. Avoid repeated freezing and thawing. Specimens showing particulate matter, erythrocytes, or turbidity must be clarified by centrifugation before testing.

INSTRUCTIONS FOR USE

PROCEDURAL NOTES

- Allow samples and reagents to reach room temperature prior to testing. Do not use water baths to thaw samples or reagents.
- Mix samples and all reagents thoroughly before use.
- Avoid excessive foaming of reagents. Also avoid exposure of reagents to excessive heat or light during storage and incubation.
- Avoid handling the tops of the wells both before and after filling.
- Standard calibrators and samples should be assayed in duplicate.
- Run a separate calibration curve for each assay.
- Use only coated wells from the same reagent batch for each assay. Also do not mix reagents from different kit lots.
- Perform incubations in a sealed box containing a wet paper towel in order to prevent evaporation.

REAGENTS PREPARATION

- Reconstitute XG-CH4 chromogen solution with 20 mL of distilled water.
- Reconstitute XG003-Calibrator with 440 μ L of distilled water for each calibrator vial.
- Prepare the required amount of XG-DB5 dilution buffer by diluting 5-fold the concentrated solution in distilled water. If crystals appear upon refrigeration, warm the bottle to 37 °C with mixing to dissolve.
- Reconstitute 1 tablet of XG-WB2 washing buffer in 500mL of distilled water.
- Prepare the required amount of XG-EA enzyme-conjugated secondary antibody solution diluting 10-fold in reconstituted XG-DB5 dilution buffer.

ASSAY PROTOCOL

1. Prepare assay reagents as described above.
2. Set up the microtiter plate with sufficient wells to enable the running of all required standards and samples.
3. Remove excess microtiter plate strips from the frame and store in the re-sealable foil bag with the desiccant provided.
4. Wash the microtiter plate strips three times with XG-WB2 washing buffer (300 μ L/well).
5. Dispense 100 μ L/well of standard calibrators (in duplicate) starting from the reconstituted solution and performing in-plate 2-fold serial dilutions in order to obtain a five-point calibration curve. Use XG-DB5 dilution buffer as diluent. For exact concentration of the reconstituted calibrator please refer to the concentration value (AU/mL) indicated on the XG003-Calibrator vial. Also dispense 100 μ L/well of XG-DB5 dilution buffer as blank, in duplicate.
6. Dispense 100 μ L/well of eight fold (1:8) diluted sample (in duplicate). Use XG-DB5 dilution buffer as diluent.
7. Incubate 1 hour at room temperature.
8. Wash six times with XG-WB2 washing buffer (300 μ L/well).
9. Add 100 μ L/well of diluted XG-EA enzyme-conjugated secondary antibody solution.
10. Incubate 1 hour at room temperature.
11. Wash six times with XG-WB2 washing buffer (300 μ L/well).
12. Prepare the required amount of chromogen-enzyme substrate solution adding 1 μ L of XG-SB enzyme substrate solution per 3 mL of XG-CH4 chromogen solution. The chromogen-enzyme substrate solution must be used within 24 hours.
13. Apply 150 μ L/well of freshly prepared chromogen-enzyme substrate solution. Allow color to develop for 20 min. at 37 °C in the dark and measure OD values of each well using an ELISA plate reader set to 405 nm. Plot the standard curve Δ OD values as described in the next section: Processing of the results.

PROCESSING OF THE RESULTS

Average the duplicate readings for each standard calibrator and sample, and subtract the zero standard optical density. The standard calibrators may be used to construct a standard curve with values reported in AU/mL (see Fig. 1). This data deduction may be performed through computer methods using curve fitting routines or may also be manually deduced by plotting the absorbance values of the standard on the y-axis versus concentration on the logarithmic x-axis and drawing the standard curve.

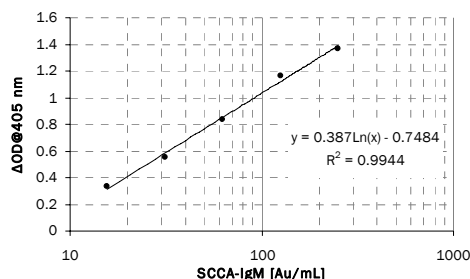


Fig. 1: Range of linearity of a typical standard curve for SCCA-IgM after 20 minutes of substrate incubation.

The immune complexes (SCCA-IgM) concentration in the biological sample can be calculated directly from the calibration curve by interpolation. The value obtained must be multiplied by the dilution factor.

QUALITY CONTROL

The intra- and inter-assay coefficients of variation were determined on 4 typical standard curves and the results were less than 15 %.

For optimal performance, the absorbance of the zero standard should be < 0.2 OD405.

It is recommended that each laboratory assays appropriate quality control samples in each run to ensure that all reagents and procedures are correct.

INTERPRETATION

The SCCA-IgM cut-off value was 120 AU/mL for differentiating HCC from non-malignant chronic liver diseases (11-14).

SPECIFIC PERFORMANCE CHARACTERISTICS

The linear range of the assay is 12,5-200 AU/mL.

Hook effect could occur for concentrations > 200 AU/mL. The sample with values above 200 AU/mL should be further diluted and re-measured.

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XEPTAGEN SpA
VEGA Science Park – Building Auriga
Via delle Industrie, 9
30175 Marghera (VE) – ITALY

Phone: +39 041 509 3910
Fax: +39 041 509 3884

info@xeptagen.com
www.xeptagen.com

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