Hepa-Ab

Antibody for the immunohistochemical detection of Hepatocellular Carcinoma (HCC) from liver biopsies

PRODUCT PROFILE
40-941-330003
FOR RESEARCH USE ONLY
Hepa-Ab

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, in particular. 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 survives 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 aetiology 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 50-70% 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 only of 60%. Prothrombin Induced by Vitamin K Absence II (PIVKA-II) or Des-γ-Carboxy Prothrombin (DCP), and glypican-3 (GPC3) serum concentrations also increase in HCC patients and are sometimes used for HCC diagnosis (5, 6). However, specificity and sensitivity is still low to give clinical significance to these assays (TAB.1) (7).

<table>
<thead>
<tr>
<th>METHODS</th>
<th>PROS</th>
<th>CONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP DETERMINATION</td>
<td>Non invasive (blood samples)</td>
<td>May lead to false positive (&gt;50 %) and false negative (&gt;30 %) results</td>
</tr>
<tr>
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<td>Non invasive (blood samples)</td>
<td>Accurate only for late stage HCC</td>
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<tr>
<td>COMPUTERIZED TOMOGRAPHY (CT SCAN)</td>
<td>Non invasive</td>
<td>Accurate when neoplastic nodules are clearly evident (&gt; 3 cm diameter)</td>
</tr>
<tr>
<td>ULTRASOUNDS (US)</td>
<td>Non invasive</td>
<td>Accurate when neoplastic nodules are clearly evident (&gt; 3 cm diameter)</td>
</tr>
<tr>
<td>MAGNETIC RESONANCE IMAGING (MRI)</td>
<td>Non invasive</td>
<td>Accurate when neoplastic nodules are clearly evident (&gt; 3 cm diameter)</td>
</tr>
<tr>
<td>HISTOLOGY (LIVER BIOPSY)</td>
<td>May confirm the diagnosis for lesions ≤2 cm</td>
<td>Invasive (liver biopsy) Expert pathologist needed</td>
</tr>
</tbody>
</table>

**TAB.1: Comparison among different current methods of HCC diagnosis.**
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 (8, 9).

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 (10).

<table>
<thead>
<tr>
<th>MARKERS</th>
<th>PROS</th>
<th>CONS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferation markers</strong> (PCNA, Ki-67, Mcm2, Mib-1)</td>
<td>Malignant grade evaluation</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Recurrence time prediction</td>
<td>Low specificity</td>
</tr>
<tr>
<td></td>
<td>Long-term survival prediction</td>
<td></td>
</tr>
<tr>
<td><strong>Nuclear morphology markers (AgNOR)</strong></td>
<td>Tumor stage evaluation</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Recurrence prediction</td>
<td>Low specificity</td>
</tr>
<tr>
<td></td>
<td>Progression prediction</td>
<td></td>
</tr>
<tr>
<td><strong>p53 and MDM2</strong></td>
<td>Long-term survival prediction</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low specificity</td>
</tr>
<tr>
<td><strong>Cell Cycle regulators</strong> (CyclinE, Cdc2, p27)</td>
<td>Recurrence time prediction</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Long-term survival prediction</td>
<td>Low specificity</td>
</tr>
<tr>
<td><strong>Tumor promoters</strong> (ras, c-myc, c-erbB-2, EGF-R)</td>
<td>Recurrence time prediction</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Progression prediction</td>
<td>Low specificity</td>
</tr>
<tr>
<td><strong>Apoptosis regulators</strong> (Fas, Fas L)</td>
<td>Recurrence time prediction</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low specificity</td>
</tr>
<tr>
<td><strong>Adhesion molecules</strong> (E-cadherin, ICAM-1, CD44 isoforms)</td>
<td>Tumor stage evaluation</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low specificity</td>
</tr>
<tr>
<td><strong>Cancer invasion markers</strong> (MMP, uPA)</td>
<td>Recurrence time prediction</td>
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<tr>
<td></td>
<td>Long-term survival prediction</td>
<td></td>
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<tr>
<td><strong>Angiogenesis promoters</strong> (VEGF, bFGF)</td>
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**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 (11).

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiofrequency thermal ablation</td>
<td>Cancer cells necrosis with radiofrequency-derived heat</td>
</tr>
<tr>
<td>Laser thermal ablation</td>
<td>Cancer cells necrosis with laser-induced heat</td>
</tr>
<tr>
<td>Percutaneous ethanol injection</td>
<td>Cancer cells necrosis with ultrasound-guided alcohol infusion</td>
</tr>
<tr>
<td>Hepatic arterial infusion</td>
<td>Cancer cells death with anti-tumoral drug infusion into hepatic artery</td>
</tr>
<tr>
<td>Chemoembolization</td>
<td>Cancer cells death with anti-tumoral drug infusion into hepatic artery, followed by blocking the flow of blood through the artery</td>
</tr>
</tbody>
</table>

**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.
Hepa-Ab
Antibody for the immunohistochemical detection of Hepatocellular Carcinoma (HCC) from liver biopsies

GenWay owns patents exploiting the discovery that, during the neoplastic transformation of the liver, a protein (Squamous Cell Carcinoma Antigen variants, SCCA) starts to be overproduced by the liver cells. In healthy subjects the presence of this protein in liver specimens is very low, almost undetectable. Patients affected by HCC overproduce this protein, and the extent of its over-expression parallels the extent of the disease. Hepa-Ab is a rabbit oligoclonal antibody generated against SCCA variants for immunohistochemical staining of liver biopsies, obtained surgically or through fine needle aspiration, providing a semi-quantitative determination of SCCA proteins expression in neoplastic liver tissue samples. Compared to conventional methods, Hepa-Ab displays the highest HCC detection sensitivity and specificity.

Hepa-Ab is a novel kit, useful for the in vitro diagnosis of HCC from liver biopsies. Kit contains lyophilized oligoclonal antibody (Hepa-Ab, oligoclonal antibody (polyclonal Ab made monospecific by affinity chromatography on HCC molecular marker columns) and dilution buffer, which can be used in immunohistochemical techniques (both manual and automatic procedures) on liver specimens (surgical or fine needle biopsies) to permit or confirm HCC diagnosis, complementing other methods. The product is developed, produced and commercialized according to ISO9001:2000 Quality Assurance Certification and is CE marked.

IMMUNOHISTOCHEMICAL AND MOLECULAR STUDIES

Liver tissues specimens were obtained, surgically or through fine needle aspiration, from:

- Patients with HCC of different aetiology such as HBV or HCV positive, HBV and HCV coinfections positive, alcohol abuse and finally without evidence of etiologic factors (totally 40 samples);
- Individuals with chronic liver diseases, such as cirrhosis (20 samples) or chronic HBV or HCV hepatitis (40 samples);
- Normal human liver (20 samples).

Tissues were embedded in paraffin section and analyzed for the presence of SCCA variants activity by immunohistochemical technique by using Hepa-Ab. After completion of staining procedures (See Hepa-Ab Manual), the percentage of stained cells in each specimen was scored on a scale of 0 to 3, in which 0 denoted negative staining, score 1 denoted reactivity in 1% - 30% of hepatocytes, score 2 denoted staining in 31% - 50% of liver cells, and score 3 denoted positive immunostaining in more than 50% of hepatocytes (FIG.1). High tissue expression of SCCA variants was detected in HCC liver biopsies with 100% sensitivity in surgical tumors, 80% in fine needle biopsies and 100% specificity when compared to normal liver used as control (12, 13, 14,15).

FIG.1: Profiles of Hepa-Ab staining on surgical liver biopsies (paraffin-embedded tissues).
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) (12-14).

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

It is important to note that liver tissues from patients affected by cirrhosis or chronic hepatitis can present low/moderate reactivity to Hepa-Ab antibody. As shown in FIG.2, only 18% of cirrhosis samples and 12% of chronic hepatitis specimens display a score 3 like staining with Hepa-Ab, versus 70% of HCC liver sections analyzed. Score 3 like Hepa-Ab immunohistochemical staining is considered highly specific of HCC (FIG.2). Hepa-Ab detects neoplastic cells independently from the HCC etiology (FIG.3).

FIG. 3: Score distribution in HCC of different etiology after Hepa-Ab staining.
The high over expression of SCCA variants was confirmed by the detection of mRNA expression using RT-PCR technique (FIG.4) (12).

**FIG.4:** SCCA variants cDNAs were achieved in frozen tumorous and non tumorous liver samples, obtained at surgery, by eminested RT-PCR analysis.

Hepa-Ab antibody may stain strongly dysplastic cells in damaged liver tissues (cirrhosis or chronic hepatitis patients). Liver cell dysplasia, developing in cirrhosis or chronic hepatitis background, has been associated with high risk of HCC onset. In fact, hepatocytes morphological/functional modifications, such as proliferative activity, increase progressively in the intermediate non-cancerous lesions, that precede HCC development. Finally, dysplasia can be considered a predictor parameter for the identification, monitoring and surveillance of high risk patients, in order to update patient’s neoplastic risk or permit a very early HCC diagnosis (16, 17). As shown in FIG.5, Hepa-Ab antibody recognizes large, multinucleate dysplastic hepatocytes positive by MIB-1 immunostaining. Hepa-Ab immunohistochemistry of liver dysplastic nodules indicated SCCA variants over-expression in 100% of cases analyzed. These data suggest that SCCA variants iper-expression may be an early event in hepatocarcinogenesis, and that Hepa-Ab may be also used as a liver pre-cancerous lesions marker.

**FIG.5:** A. Strongly cytoplasmic Hepa-Ab positive staining of mostly dysplastic hepatocytes (50X magnification); B. Positive immunostaining with MIB-1 antibody of dysplastic area (50X magnification).
Further studies carried out by Giannelli et al. suggested that SCCA could be useful as a tissue marker for the detection of HCC and metastatic nodules (13,15). In the study, carried out on 52 patients affected by HCC and 48 cirrhotic patients, SCCA antigen appeared strongly stained in all of the HCC specimens, the pattern of expression being irregular and spotty. In cancer cells, SCCA was localized mainly in the cytoplasm with a granular pattern. These findings were consistent in all cases, and no substantial differences (p > 0.1) were observed regarding different HCC’s. The SCCA antigen was more abundantly expressed in HCC than in peritumoral cirrhotic tissue, $1,308.1 \pm 599.7$ and $469.8 \pm 313.4 \mu m^2$, respectively, this difference being statistically significant (p < 0.001).

However, in some peritumoral tissue, intense SCCA expression was observed in correspondence with histologically ascertained metastatic HCC nodules. This supports the contention that SCCA was more abundantly expressed in HCC than in the peritumoral cirrhotic tissue (Fig. 6).

No correlation of antigen levels in tissue with tumor size, HCC histological degree, and survival was found (tissue/tumor size: $r = -0.05$; p > 0.1 and $r = -0.04$; p > 0.1, respectively; tissue/histology degree: $r = -0.13$; p > 0.1 and $r = 0.10$; p > 0.1, respectively; tissue/survival: $r = 0.11$; p > 0.1 and $r = -0.01$; p > 0.1, respectively).

**FIG. 6:** SCCA Expression in HCC and peritumoral tissue. Expression of the SCCA is stronger in HCC (b) than in peritumoral cirrhotic tissue (a). Strong expression is also observed in metastatic nodules (c). At higher magnification, the SCCA appears localized mainly intracellularly in peritumoral cirrhotic (d) in HCC (e) and in a metastatic nodule (Mtx) present in peritumoral cirrhotic (LC) tissue (f). Scale bar = 20 μm.
SELECTED Hepa-Ab IMMUNOSTAINING FIGURES

**FIG. 7**: SCCA variants overexpression (Score 1) in well differentiated HCC (20X magnification). Part of HCC shows a well limited focus of SCCA variants expressing tumoral cells. Positive hepatocytes show a weak diffuse cytoplasmatic labelling.

**FIG. 8**: SCCA variants expression (Score 2) in moderately differentiated HCC (clear cell type) (30X magnification). Variation of staining from one area to another is well visible. Note as labelling is confined at membrane area in clear cells.

**FIG. 9**: SCCA variants expression (Score 3) in poorly differentiated hepatocellular carcinoma (60X magnification).
**FIG.10**: Other examples of Hepa-Ab immunostaining of surgically obtained liver specimens (20X, 25X, 60X magnification, respectively).

**FIG.11**: A. Hematoxylin-Eosin staining of surgical liver cancer specimen (trabecular/acinar type, area on the right) (16X magnification). B. Hepa-Ab immunostaining of the same liver section of panel A (16X magnification): note the intense, cytoplasmic, score 3 like staining of cancerous area on the right.

**FIG.12**: A. Hepa-Ab staining of fine needle aspiration obtained HCC specimen (Score 3, 50X magnification): note the intense, cytoplasmic, score 3 like staining. B. MIB-1 staining of fine needle aspiration obtained HCC specimen (50X magnification).
REFERENCES

7. Fujiyama S. et al., Oncology 62(suppl 1):57-63, 2002
Hepa-Ab

*An immunohistochemical kit for the detection of Hepatocellular Carcinoma (HCC) from liver biopsies*

(100 determinations)

**INSTRUCTIONS FOR USE**
Hepa-Ab: Instructions for use

**PRODUCT CODE**  Hepa-Ab XG001

**INTENDED USE**  Hepa-Ab contains an oligoclonal antibody anti-Squamous Cell Carcinoma antigen (anti-SCCA) variants, providing qualitative and semi-quantitative demonstrations of SCCA variants (SCCA-1, SCCA-2, SCCA-PD) in formalin-fixed, paraffin-embedded human liver tissue sections (1) or cryostat human liver sections.

**EXPLANATION**  Hepa-Ab is an innovative immunohistochemical kit for the diagnosis of Hepatocellular Carcinoma (HCC) in surgical and fine-needle biopsies based on the detection of SCCA variants (1,2,3). High levels of SCCA variants have been detected in all HCC surgical tumors, with no reactivity detectable in normal human livers (1,2). Over-expression of SCCA variants has indicated the pathological stages of HCC. No correlation due to etiology was found (1,2). Moreover, expression levels of SCCA have been shown to be significantly higher in tumoral tissue than in cirrhotic peritumoral tissue (2,4,5). Hepa-Ab has also been used to determine the expression of SCCA variants in squamous cell carcinomas (6).

**PRINCIPLE OF THE TEST**  Specific immunostaining is accomplished by localizing the SCCA variants antigens with Hepa-Ab by the use of the avidin-biotin peroxidase (ABC) method (7). Specifically, with this method a biotinylated secondary antibody binds to the primary antibody that is complexed with any SCCA variants located in the tissue. A chromogen solution is then added forming a colored deposit (usually brown) in the presence of the avidin-biotin peroxidase complex, thus revealing the SCCA variants. The sample is counterstained and a coverslip is mounted to ensure best viewing. Results are interpreted using a light microscope. The interpretation of immunostaining results must be complemented with the use of positive and negative controls to ensure correct methodology.

**REAGENTS AND MATERIALS PROVIDED**
- **XG001**: Lyophilized rabbit anti-Squamous Cell Carcinoma antigen (anti-SCCA) variants from a solution containing 20mM phosphate pH 7.2
- **Reconstitution buffer**: 1 mL phosphate buffer

**STORAGE**  Avoid repeated freeze and thaw cycles
- Unopened kit may be stored at 4°C.
- Once resuspended, the antibody may be stored at 4°C for short-term only.
- For long-term storage, store resuspended antibody in aliquots at -20°C.
- The Prepare Working Dilutions only on the day of use.

**EXPIRATION DATE**  See label on vial.

**CONTROLS**  **POSITIVE TISSUE CONTROL**: Epidermic and follicular area
1. Non-patient tissue containing antigen to be detected
2. Tissue fixed and processed in the same way as patient sample

Objective:
1. Control all steps of the analysis
2. Training of user for the appearance of positive reaction
3. Semiquantitative comparison of the reaction
NEGATIVE TISSUE CONTROL: Dermic area
1. Tissue that does not express the antigen
2. Tissue that is fixed and processed in the same way as patient sample

Objective:
1. Detects of unintended antibody cross-reactivity
2. Provides the absence of specific immunostaining
3. Provides an indication of non-specific background staining

INSTRUCTIONS FOR USE

Reagents preparation - Resuspend Hepa-Ab antibody by adding 1,0 mL of the Reconstitution Buffer provided and gently agitate the vial with its top securely attached in order to dissolve any antibody that may be under the cap. Resuspension should occur at least 1 hour before use and vial should be allowed to sit at 4°C in order to dissolve the lyophil completely and to secure its equal distribution in the buffer.

Protocol
1. De-Paraffinate tissue sections by incubation in xylene for 30 minutes
2. Hydrate by placing in 100%, 95%, 70%, 50% ethanol
3. Wash with distilled water
4. Inactivate the endogenous peroxidase by incubation for 10 minutes in 3% hydrogen peroxide in PBS.
5. Wash with PBS containing 0.1% Tween.
6. Perform antigen unmasking by placing the slides with samples in a 0.01 M citrate buffer pH 6 bath and heating in microwave. We suggest performing 3 cycles of 5 minutes each at 750 Watt(*).
7. Wash with PBS containing 0.1% Tween.
8. Block slides with animal serum taken from the same secondary antibody animal species. Incubate for 10 minutes at room temperature in a humidified chamber.
9. Remove residual liquid.
10. Incubate slides in a humidified chamber for 1 hour at room temperature with a Hepa-Ab dilution range of 1:5 – 1:10 in PBS containing 0.1% Tween (*). Special attention should be taken in order to avoid drying out of the sample.
11. Wash with PBS containing 0.1% Tween.
12. Incubate with biotinylated secondary antibody for 10 min at room temperature in a humidified chamber diluted as described by manufacturer.
13. Wash with PBS containing 0.1% Tween.
14. Incubate with ABC complex for 10 min at room temperature.
15. Wash with PBS containing 0.1% Tween.
16. Incubate with chromogen solution (DAB) for maximum 5 minutes. Viewing of the sample under the microscope may help determine a correct incubation time to avoid overstaining (*).
17. Wash with distilled water.
18. Counterstain with hematoxylin solution for 1 minute (**).
19. Wash with warm tap water.
20. Dehydrate slides in 80%, 90% and 2x 100% ethanol.
21. Immerse in 2 changes of xylene.
22. Mount cover slips on the slides and examine under a light microscope.

(*) These are guidelines only; each lab should determine optimal dilution and incubation time.
(**) When interpreting an IHC result, the morphology of each tissue sample should be examined by hematoxylin and eosin (H&E) staining.

SUMMARY OF EXPECTED
Hepa-Ab stains predominantly the cytoplasm of the liver cancer cells (1, 2).
IMMUNOSTAINING

INTERPRETATION

The immunostaining intensity will reflect not only the effects of tissue preparation, but especially antigen concentration. An intense immunostaining indicates a relatively high concentration of HCC molecular marker, while lighter immunostaining will be indicative of a lower concentration. It should be helpful to score the percentage of stained cells in each specimen as follows (1):

- Score 0: Denotes negative staining
- Score 1: positivity in 1-30%
- Score 2: positivity in 31-50%
- Score 3: positivity in more than 50%

TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative/Low staining on positive tissue</td>
<td>1. Procedure may not have been run properly</td>
</tr>
<tr>
<td></td>
<td>2. Procedure steps were omitted or performed in the wrong order</td>
</tr>
<tr>
<td></td>
<td>3. Deparaffinization was not complete</td>
</tr>
<tr>
<td></td>
<td>4. Degradation of antigens</td>
</tr>
<tr>
<td></td>
<td>5. The primary antibody may be too diluted</td>
</tr>
<tr>
<td></td>
<td>6. The detection system reagents may be too diluted</td>
</tr>
<tr>
<td></td>
<td>7. Incubation times of the detection system reagents may be too short</td>
</tr>
<tr>
<td></td>
<td>8. Counterstain or coverslip mounting is incompatible with chromogen</td>
</tr>
<tr>
<td></td>
<td>9. Antigen unmasking may not have been done properly</td>
</tr>
<tr>
<td></td>
<td>10. Tissue may be not properly prepared or may be overfixed</td>
</tr>
<tr>
<td>Non-specific background staining</td>
<td>1. Protein blocking step was ineffective</td>
</tr>
<tr>
<td></td>
<td>2. Endogenous enzyme activity</td>
</tr>
<tr>
<td></td>
<td>3. Deparaffinization was not complete</td>
</tr>
<tr>
<td></td>
<td>4. Reagent dried on tissue during immunostaining</td>
</tr>
<tr>
<td></td>
<td>5. Primary antibody or detection system reagents are too concentrated or incubation times are too long</td>
</tr>
<tr>
<td></td>
<td>6. Overdevelopment of chromogen</td>
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</tbody>
</table>

BIBLIOGRAPHY

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<td><strong>COMPUTERIZED TOMOGRAPHY (CT) SCAN</strong></td>
<td>Non invasive</td>
<td>Accurate when neoplastic nodules are clearly evident (&gt; 3 cm diameter)</td>
</tr>
<tr>
<td><strong>ULTRASOUNDS (US)</strong></td>
<td>Non invasive</td>
<td>Accurate when neoplastic nodules are clearly evident (&gt; 3 cm diameter)</td>
</tr>
<tr>
<td><strong>MAGNETIC RESONANCE IMAGING (MRI)</strong></td>
<td>Non invasive</td>
<td>Accurate when neoplastic nodules are clearly evident (&gt; 3 cm diameter)</td>
</tr>
<tr>
<td><strong>HISTOLOGY (LIVER BIOPSY)</strong></td>
<td>May confirm the diagnosis for lesions ( \leq 2 ) cm</td>
<td>Invasive (liver biopsy) Expert pathologist needed</td>
</tr>
</tbody>
</table>

**TAB.1**: Comparison among different current methods of HCC diagnosis.
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 (8, 9).

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 (10).

<table>
<thead>
<tr>
<th>MARKERS</th>
<th>PROS</th>
<th>CONS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferation markers (PCNA, Ki-67, Mcm2, Mib-1)</strong></td>
<td>Malignant grade evaluation</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Recurrence time prediction</td>
<td>Low specificity</td>
</tr>
<tr>
<td></td>
<td>Long-term survival prediction</td>
<td></td>
</tr>
<tr>
<td><strong>Nuclear morphology markers (AgNOR)</strong></td>
<td>Tumor stage evaluation</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Recurrence prediction</td>
<td>Low specificity</td>
</tr>
<tr>
<td></td>
<td>Progression prediction</td>
<td></td>
</tr>
<tr>
<td><strong>p53 and MDM2</strong></td>
<td>Long-term survival prediction</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low specificity</td>
</tr>
<tr>
<td><strong>Cell Cycle regulators (CyclinE, Cdc2, p27)</strong></td>
<td>Recurrence time prediction</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Long-term survival prediction</td>
<td>Low specificity</td>
</tr>
<tr>
<td><strong>Tumor promoters (ras, c-myc, c-erbB-2, EGF-R)</strong></td>
<td>Recurrence time prediction</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Progression prediction</td>
<td>Low specificity</td>
</tr>
<tr>
<td><strong>Apoptosis regulators (Fas, Fas L)</strong></td>
<td>Recurrence time prediction</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low specificity</td>
</tr>
<tr>
<td><strong>Adhesion molecules (E-cadherin, ICAM-1, CD44 isoforms)</strong></td>
<td>Tumor stage evaluation</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low specificity</td>
</tr>
<tr>
<td><strong>Cancer invasion markers (MMP, uPA)</strong></td>
<td>Recurrence time prediction</td>
<td>Low specificity</td>
</tr>
<tr>
<td></td>
<td>Long-term survival prediction</td>
<td></td>
</tr>
<tr>
<td><strong>Angiogenesis promoters (VEGF, bFGF)</strong></td>
<td>Long-term survival prediction</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low specificity</td>
</tr>
</tbody>
</table>

**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 (11).

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiofrequency thermal ablation</td>
<td>Cancer cells necrosis with radiofrequency-derived heat</td>
</tr>
<tr>
<td>Laser thermal ablation</td>
<td>Cancer cells necrosis with laser-induced heat</td>
</tr>
<tr>
<td>Percutaneous ethanol injection</td>
<td>Cancer cells necrosis with ultrasound-guided alcohol infusion</td>
</tr>
<tr>
<td>Hepatic arterial infusion</td>
<td>Cancer cells death with anti-tumoral drug infusion into hepatic artery</td>
</tr>
<tr>
<td>Chemoembolization</td>
<td>Cancer cells death with anti-tumoral drug infusion into hepatic artery, followed by blocking the flow of blood through the artery</td>
</tr>
</tbody>
</table>

**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.*
Hepa-Ab
Antibody for the immunohistochemical detection of Hepatocellular Carcinoma (HCC) from liver biopsies

GenWay owns patents exploiting the discovery that, during the neoplastic transformation of the liver, a protein (Squamous Cell Carcinoma Antigen variants, SCCA) starts to be overproduced by the liver cells. In healthy subjects the presence of this protein in liver specimens is very low, almost undetectable. Patients affected by HCC overproduce this protein, and the extent of its over-expression parallels the extent of the disease. Hepa-Ab is a rabbit oligoclonal antibody generated against SCCA variants for immunohistochemical staining of liver biopsies, obtained surgically or through fine needle aspiration, providing a semi-quantitative determination of SCCA proteins expression in neoplastic liver tissue samples. Compared to conventional methods, Hepa-Ab displays the highest HCC detection sensitivity and specificity.

Hepa-Ab is a novel kit, useful for the in vitro diagnosis of HCC from liver biopsies. Kit contains lyophilized oligoclonal antibody (Hepa-Ab, oligoclonal antibody (polyclonal Ab made monospecific by affinity chromatography on HCC molecular marker columns) and dilution buffer, which can be used in immunohistochemical techniques (both manual and automatic procedures) on liver specimens (surgical or fine needle biopsies) to permit or confirm HCC diagnosis, complementing other methods. The product is developed, produced and commercialized according to ISO9001:2000 Quality Assurance Certification and is CE marked.

IMMUNOHISTOCHEMICAL AND MOLECULAR STUDIES

Liver tissues specimens were obtained, surgically or through fine needle aspiration, from:

- Patients with HCC of different aetiology such as HBV or HCV positive, HBV and HCV coinfections positive, alcohol abuse and finally without evidence of etiologic factors (totally 40 samples);
- Individuals with chronic liver diseases, such as cirrhosis (20 samples) or chronic HBV or HCV hepatitis (40 samples);
- Normal human liver (20 samples).

Tissues were embedded in paraffin section and analyzed for the presence of SCCA variants activity by immunohistochemical technique by using Hepa-Ab. After completion of staining procedures (See Hepa-Ab Manual), the percentage of stained cells in each specimen was scored on a scale of 0 to 3, in which 0 denoted negative staining, score 1 denoted reactivity in 1% - 30% of hepatocytes, score 2 denoted staining in 31% - 50% of liver cells, and score 3 denoted positive immunostaining in more than 50% of hepatocytes (FIG.1). High tissue expression of SCCA variants was detected in HCC liver biopsies with 100% sensitivity in surgical tumors, 80% in fine needle biopsies and 100% specificity when compared to normal liver used as control (12, 13, 14,15).

![Profile of Hepa-Ab staining on surgical liver biopsies (paraffin-embedded tissues).](FIG.1)
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) (12-14).

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

It is important to note that liver tissues from patients affected by cirrhosis or chronic hepatitis can present low/moderate reactivity to Hepa-Ab antibody. As shown in FIG.2, only 18% of cirrhosis samples and 12% of chronic hepatitis specimens display a score 3 like staining with Hepa-Ab, versus 70% of HCC liver sections analyzed. Score 3 like Hepa-Ab immunohistochemical staining is considered highly specific of HCC (FIG.2). Hepa-Ab detects neoplastic cells independently from the HCC etiology (FIG.3).

**FIG. 3:** Score distribution in HCC of different etiology after Hepa-Ab staining.
The high over expression of SCCA variants was confirmed by the detection of mRNA expression using RT-PCR technique (FIG.4) (12).

**FIG.4:** SCCA variants cDNAs were achieved in frozen tumorous and non tumorous liver samples, obtained at surgery, by eminested RT-PCR analysis.

Hepa-Ab antibody may stain strongly dysplastic cells in damaged liver tissues (cirrhosis or chronic hepatitis patients). Liver cell dysplasia, developing in cirrhosis or chronic hepatitis background, has been associated with high risk of HCC onset. In fact, hepatocytes morphological/functional modifications, such as proliferative activity, increase progressively in the intermediate non-cancerous lesions, that precede HCC development. Finally, dysplasia can be considered a predictor parameter for the identification, monitoring and surveillance of high risk patients, in order to update patient’s neoplastic risk or permit a very early HCC diagnosis (16, 17). As shown in FIG.5, Hepa-Ab antibody recognizes large, multinucleate dysplastic hepatocytes positive by MIB-1 immunostaining. Hepa-Ab immunohistochemistry of liver dysplastic nodules indicated SCCA variants over-expression in 100% of cases analyzed. These data suggest that SCCA variants iper-expression may be an early event in hepatocarcinogenesis, and that Hepa-Ab may be also used as a liver pre-cancerous lesions marker.

**FIG.5:** A. Strongly cytoplasmic Hepa-Ab positive staining of mostly dysplastic hepatocytes (50X magnification); B. Positive immunostaining with MIB-1 antibody of dysplastic area (50X magnification).
Further studies carried out by Giannelli et al. suggested that SCCA could be useful as a tissue marker for the detection of HCC and metastatic nodules (13,15). In the study, carried out on 52 patients affected by HCC and 48 cirrhotic patients, SCCA antigen appeared strongly stained in all of the HCC specimens, the pattern of expression being irregular and spotty. In cancer cells, SCCA was localized mainly in the cytoplasm with a granular pattern. These findings were consistent in all cases, and no substantial differences (p > 0.1) were observed regarding different HCC’s. The SCCA antigen was more abundantly expressed in HCC than in peritumoral cirrhotic tissue, 1,308.1 ± 599.7 and 469.8 ± 313.4 μm², respectively, this difference being statistically significant (p < 0.001). However, in some peritumoral tissue, intense SCCA expression was observed in correspondence with histologically ascertained metastatic HCC nodules. This supports the contention that SCCA was more abundantly expressed in HCC than in the peritumoral cirrhotic tissue (Fig. 6).

No correlation of antigen levels in tissue with tumor size, HCC histological degree, and survival was found (tissue/tumor size: r = - 0.05; p >0.1 and r = - 0.04; p >0.1, respectively; tissue/histology degree: r = - 0.13; p >0.1 and r = 0.10; p >0.1, respectively; tissue/survival: r = 0.11; p >0.1 and r = - 0.01; p >0.1, respectively).

**FIG.6:** SCCA Expression in HCC and peritumoral tissue. Expression of the SCCA is stronger in HCC (b) than in peritumoral cirrhotic tissue (a). Strong expression is also observed in metastatic nodules (c). At higher magnification, the SCCA appears localized mainly intracellularly in peritumoral cirrhotic (d) in HCC (e) and in a metastatic nodule (Mtx) present in peritumoral cirrhotic (LC) tissue (f). Scale bar = 20 μm.
SELECTED Hepa-Ab IMMUNOSTAINING FIGURES

**FIG.7**: SCCA variants overexpression (Score 1) in well differentiated HCC (20X magnification). Part of HCC shows a well limited focus of SCCA variants expressing tumoral cells. Positive hepatocytes show a weak diffuse cytoplasmatic labelling.

**FIG.8**: SCCA variants expression (Score 2) in moderately differentiated HCC (clear cell type) (30X magnification). Variation of staining from one area to another is well visible. Note as labelling is confined at membrane area in clear cells.

**FIG.9**: SCCA variants expression (Score 3) in poorly differentiated hepatocellular carcinoma (60X magnification).
**FIG.10**: Other examples of Hepa-Ab immunostaining of surgically obtained liver specimens (20X, 25X, 60X magnification, respectively).

**FIG.11**:  
A. Hematoxylin-Eosin staining of surgical liver cancer specimen (trabecular/acinar type, area on the right) (16X magnification).  
B. Hepa-Ab immunostaining of the same liver section of panel A (16X magnification): note the intense, cytoplasmic, score 3 like staining of cancerous area on the right.

**FIG.12**:  
A. Hepa-Ab staining of fine needle aspiration obtained HCC specimen (Score 3, 50X magnification): note the intense, cytoplasmic, score 3 like staining.  
B. MIB-1 staining of fine needle aspiration obtained HCC specimen (50X magnification).
REFERENCES

7. Fujiyama S. et al., Oncology 62(suppl 1):57-63, 2002
Hepa-Ab

An immunohistochemical kit for the detection of Hepatocellular Carcinoma (HCC) from liver biopsies
(100 determinations)

INSTRUCTIONS FOR USE
Hepa-Ab: Instructions for use

PRODUCT CODE  Hepa-Ab XG001

INTENDED USE  Hepa-Ab contains an oligoclonal antibody anti-Squamous Cell Carcinoma antigen (anti-SCCA) variants, providing qualitative and semi-quantitative demonstrations of SCCA variants (SCCA-1, SCCA-2, SCCA-PD) in formalin-fixed, paraffin-embedded human liver tissue sections (1) or cryostat human liver sections.

EXPLANATION  Hepa-Ab is an innovative immunohistochemical kit for the diagnosis of Hepatocellular Carcinoma (HCC) in surgical and fine-needle biopsies based on the detection of SCCA variants (1,2,3). High levels of SCCA variants have been detected in all HCC surgical tumors, with no reactivity detectable in normal human livers (1,2). Over-expression of SCCA variants has indicated the pathological stages of HCC. No correlation due to etiology was found (1,2). Moreover, expression levels of SCCA have been shown to be significantly higher in tumoral tissue than in cirrhotic peritumoral tissue (2,4,5). Hepa-Ab has also been used to determine the expression of SCCA variants in squamous cell carcinomas (6).

PRINCIPLE OF THE TEST  Specific immunostaining is accomplished by localizing the SCCA variants antigens with Hepa-Ab by the use of the avidin-biotin peroxidase (ABC) method (7). Specifically, with this method a biotinylated secondary antibody binds to the primary antibody that is complexed with any SCCA variants located in the tissue. A chromogen solution is then added forming a colored deposit (usually brown) in the presence of the avidin-biotin peroxidase complex, thus revealing the SCCA variants. The sample is counterstained and a coverslip is mounted to ensure best viewing. Results are interpreted using a light microscope. The interpretation of immunostaining results must be complemented with the use of positive and negative controls to ensure correct methodology.

REAGENTS AND MATERIALS PROVIDED  - XG001: lyophilized rabbit anti-Squamous Cell Carcinoma antigen (anti-SCCA) variants from a solution containing 20mM phosphate pH 7.2
- Reconstitution buffer: 1 mL phosphate buffer

STORAGE  Avoid repeated freeze and thaw cycles
- Unopened kit may be stored at 4°C.
- Once resuspended, the antibody may be stored at 4°C for short-term only.
- For long-term storage, store resuspended antibody in aliquots at -20°C.
- The Prepare Working Dilutions only on the day of use.

EXPIRATION DATE  SEE LABEL ON VIAL.

CONTROLS  POSITIVE TISSUE CONTROL: Epidermic and follicular area
  1. Non-patient tissue containing antigen to be detected
  2. Tissue fixed and processed in the same way as patient sample
Objective:
  1. Control all steps of the analysis
  2. Training of user for the appearance of positive reaction
  3. Semiquantitative comparison of the reaction
NEGATIVE TISSUE CONTROL: Dermic area

1. Tissue that does not express the antigen
2. Tissue that is fixed and processed in the same way as patient sample

Objective:
1. Detects of unintended antibody cross-reactivity
2. Provides the absence of specific immunostaining
3. Provides an indication of non-specific background staining

INSTRUCTIONS FOR USE

Reagents preparation
- Resuspend Hepa-Ab antibody by adding 1,0 mL of the Reconstitution Buffer provided and gently agitate the vial with its top securely attached in order to dissolve any antibody that may be under the cap. Resuspension should occur at least 1 hour before use and vial should be allowed to sit at 4°C in order to dissolve the lyophil completely and to secure its equal distribution in the buffer.

Protocol
1. De-Paraffinate tissue sections by incubation in xylene for 30 minutes
2. Hydrate by placing in 100%, 95%, 70%, 50% ethanol
3. Wash with distilled water
4. Inactivate the endogenous peroxidase by incubation for 10 minutes in 3% hydrogen peroxide in PBS.
5. Wash with PBS containing 0.1% Tween.
6. Perform antigen unmasking by placing the slides with samples in a 0.01 M citrate buffer pH 6 bath and heating in microwave. We suggest performing 3 cycles of 5 minutes each at 750 Watt(*).
7. Wash with PBS containing 0.1% Tween.
8. Block slides with animal serum taken from the same secondary antibody animal species. Incubate for 10 minutes at room temperature in a humidified chamber.
9. Remove residual liquid.
10. Incubate slides in a humidified chamber for 1 hour at room temperature with a Hepa-Ab dilution range of 1:5 – 1:10 in PBS containing 0.1% Tween (*). Special attention should be taken in order to avoid drying out of the sample.
11. Wash with PBS containing 0.1% Tween
12. Incubate with biotinylated secondary antibody for 10 min at room temperature in a humidified chamber diluted as described by manufacturer
13. Wash with PBS containing 0.1% Tween.
14. Incubate with ABC complex for 10 min at room temperature
15. Wash with PBS containing 0.1% Tween.
16. Incubate with chromogen solution (DAB) for maximum 5 minutes. Viewing of the sample under the microscope may help determine a correct incubation time to avoid overstaining (*)
17. Wash with distilled water
18. Counterstain with hematoxylin solution for 1 minute (**)
19. Wash with warm tap water
20. Dehydrate slides in 80%, 90% and 2x 100% ethanol
21. Immerse in 2 changes of xylene
22. Mount cover slips on the slides and examine under a light microscope

(*) These are guidelines only; each lab should determine optimal dilution and incubation time.
(**) When interpreting an IHC result, the morphology of each tissue sample should be examined by hematoxylin and eosin (H&E) staining.

SUMMARY OF EXPECTED
Hepa-Ab stains predominantly the cytoplasm of the liver cancer cells (1, 2).
IMMUNOSTAINING

INTERPRETATION

The immunostaining intensity will reflect not only the effects of tissue preparation, but especially antigen concentration. An intense immunostaining indicates a relatively high concentration of HCC molecular marker, while lighter immunostaining will be indicative of a lower concentration. It should be helpful to score the percentage of stained cells in each specimen as follows (1):

- Score 0: Denotes negative staining
- Score 1: positivity in 1-30%
- Score 2: positivity in 31-50%
- Score 3: positivity in more than 50%

TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative/Low staining on positive tissue</td>
<td>1. Procedure may not have been run properly</td>
</tr>
<tr>
<td></td>
<td>2. Procedure steps were omitted or performed in the wrong order</td>
</tr>
<tr>
<td></td>
<td>3. Deparaffinization was not complete</td>
</tr>
<tr>
<td></td>
<td>4. Degradation of antigens</td>
</tr>
<tr>
<td></td>
<td>5. The primary antibody may be too diluted</td>
</tr>
<tr>
<td></td>
<td>6. The detection system reagents may be too diluted</td>
</tr>
<tr>
<td></td>
<td>7. Incubation times of the detection system reagents may be too short</td>
</tr>
<tr>
<td></td>
<td>8. Counterstain or coverslip mounting is incompatible with chromogen</td>
</tr>
<tr>
<td></td>
<td>9. Antigen unmasking may not have been done properly</td>
</tr>
<tr>
<td></td>
<td>10. Tissue may be not properly prepared or may be overfixed</td>
</tr>
<tr>
<td>Non-specific background staining</td>
<td>1. Protein blocking step was ineffective</td>
</tr>
<tr>
<td></td>
<td>2. Endogenous enzyme activity</td>
</tr>
<tr>
<td></td>
<td>3. Deparaffinization was not complete</td>
</tr>
<tr>
<td></td>
<td>4. Reagent dried on tissue during immunostaining</td>
</tr>
<tr>
<td></td>
<td>5. Primary antibody or detection system reagents are too concentrated or incubation times are too long</td>
</tr>
<tr>
<td></td>
<td>6. Overdevelopment of chromogen</td>
</tr>
</tbody>
</table>

BIBLIOGRAPHY