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**DEVELOPMENT OF THREE MONOCLONAL ANTIBODIES (AFP,
DCP, TXN) AND THEIR PRELIMINARY APPLICATION IN THE
DETECTION OF HEPATOCELLULAR CARCINOMA**

SUMMARY OF DISSERTATION ON APPLIED BIOLOGY

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The dissertation can be accessed at:

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INTRODUCTION

Rationale of the Dissertation:

Liver cancer is currently a major global health challenge, with rapidly increasing incidence and mortality rates. This disease imposes a heavy burden on healthcare systems, especially in developing countries. Among primary liver cancers, hepatocellular carcinoma (HCC) accounts for approximately 90% of all cases. The pathogenesis of HCC involves multiple mechanisms, including genetic factors; infection with hepatitis B or C viruses; chronic alcohol consumption; exposure to toxic substances; and metabolic disorders—leading to molecular alterations and carcinogenesis.

The selection of the three biomarkers AFP, DCP, and TXN stems from the urgent need for early and accurate detection of HCC, especially since AFP alone lacks sufficient sensitivity and specificity. AFP is a commonly used biomarker but has limited sensitivity (only about 70% of cases). Meanwhile, DCP has been shown to significantly improve specificity, supporting disease detection even when AFP is negative or at low levels. TXN is a new biomarker linked to tumor progression, apoptosis, and invasion, adding value to the early detection of HCC that AFP and DCP may miss. Given this practical need, research into creating specific monoclonal antibodies against these three biomarkers, AFP, DCP, and TXN, is of significant importance. Monoclonal antibodies are not only a key tool in modern immunological techniques, but also form the basis for developing test kits with wide applicability in the early detection of hepatocellular carcinoma. Therefore, this dissertation was conducted under the title: *“Development of Three Monoclonal Antibodies (AFP, DCP, TXN) and Their Preliminary Application in the Detection of Hepatocellular Carcinoma.”*

Research Objectives:

1. To produce recombinant antigens corresponding to the three hepatocellular carcinoma biomarkers AFP, DCP, and TXN.

2. To generate monoclonal antibodies specific to AFP, DCP, and TXN antigens derived from the recombinant antigens produced.
3. To conduct preliminary applications of the generated monoclonal antibodies for the detection of hepatocellular carcinoma..

Novel Contributions:

- Successfully generated a panel of three monoclonal antibodies against recombinant antigens corresponding to the biomarkers AFP, DCP, and TXN for diagnostic application in hepatocellular carcinoma.
- Applied the developed monoclonal antibodies in preliminary studies on HCC detection, and evaluated their combined diagnostic performance in terms of sensitivity and specificity.

Scientific and Practical Significance:

- Scientific significance: The findings demonstrate mastery of a complete biotechnological workflow—from bioinformatics analysis, immunogenic epitope design, recombinant antigen expression, and hybridoma generation to antigen detection in serum samples. This represents a significant contribution to the foundational research on cancer diagnostic bioproducts in Vietnam, advancing technological autonomy and reducing dependence on imported reagents.
- Practical significance: The study opens new prospects for developing a domestic ELISA-based multi-marker diagnostic kit utilizing locally produced monoclonal antibodies, thereby improving the detection capability for hepatocellular carcinoma, particularly in AFP-negative cases. The resulting products can be trialed in provincial hospitals and potentially implemented widely in community screening programs, especially in regions with high HCC prevalence.

Chapter 1. LITERATURE REVIEW

1.1. OVERVIEW OF HEPATOCELLULAR CARCINOMA (HCC)

Liver cancer can be classified into two major types: secondary (metastatic) liver cancer and primary liver cancer. Secondary liver cancer occurs when malignant cells originating from other organs or tissues migrate via the bloodstream or lymphatic system to the liver, establish there, and form tumors. Primary liver cancer, on the other hand, is a malignant disease arising within the liver itself, in which normal hepatocytes undergo morphological and functional transformation into cancer cells. Primary liver cancers are divided into three main histological types: HCC, intrahepatic cholangiocarcinoma, and hepatoblastoma. Among these, HCC is by far the most common, accounting for approximately 90% of all primary liver cancers.

1.1.1. Epidemiology of Hepatocellular Carcinoma

HCC is the most prevalent form of primary liver cancer, accounting for about 80–90% of all liver cancer cases. Globally, HCC ranks sixth in incidence and fourth in cancer-related mortality, with approximately 866,000 new cases and 759,000 deaths annually. The disease burden is highest in East Asia and sub-Saharan Africa, primarily due to the high prevalence of chronic hepatitis B virus (HBV) infection. Mongolia currently has the world's highest incidence rate of HCC. Although age-standardized incidence rates are declining as a result of HBV vaccination programs and HCV treatment, the absolute number of cases continues to rise because of population aging. Countries that previously had low HCC incidence—such as the United States, Iran, and Nepal—are now experiencing an upward trend, largely attributed to chronic hepatitis C infection and non-alcoholic fatty liver disease (NAFLD). In Vietnam, HCC remains one of the most common and fatal malignancies, with over 24,000 new cases and 25,000 deaths reported annually. The incidence rate of HCC in Vietnam is nearly three times higher than the global average, mainly due to chronic hepatitis B infection. Despite long-standing national

HBV immunization programs, their effectiveness has been limited in adults who were already infected before vaccine implementation. Consequently, Vietnam is currently among the countries with the highest global HCC burden and is a priority nation in the World Health Organization's strategy for viral hepatitis elimination by 2030.

1.1.2. Pathogenetic Factors of HCC

The pathogenesis of hepatocellular carcinoma involves complex interactions between genetic and environmental factors. From a genetic perspective, key somatic mutations such as TERT (44–60%), TP53 (30–50%), CTNNB1 (20–40%), and others including AXIN1, ARID1A, ARID2, and NFE2L2 contribute to hepatocarcinogenesis through dysregulation of critical molecular pathways such as telomerase transcription, Wnt/ β -catenin signaling, DNA repair, and oxidative stress response. Additionally, several germline polymorphisms—including PNPLA3, TM6SF2, and MBOAT7—have been linked to increased HCC susceptibility, particularly through mechanisms associated with non-alcoholic steatohepatitis (NASH) and liver fibrosis. Among environmental factors, HBV infection remains the dominant cause in Asia and Africa, capable of inducing HCC even in the absence of cirrhosis due to viral DNA integration into the host genome. HCV infection, which predominates in developed countries, leads to HCC mainly through chronic inflammation and cirrhosis. Chronic alcohol consumption significantly increases HCC risk, especially when combined with HBV infection. Moreover, metabolic factors such as non-alcoholic fatty liver disease (NAFLD), particularly non-alcoholic steatohepatitis (NASH), type 2 diabetes mellitus, obesity, and insulin resistance are increasingly recognized as major contributors to hepatocarcinogenesis—particularly in industrialized nations. These conditions act primarily through insulin/IGF-1 signaling pathways and chronic hepatic inflammation, which promote cellular proliferation and malignant transformation.

1.2. CURRENTLY USED METHODS AND THE NEED FOR NEW APPROACHES IN THE DIAGNOSIS OF HEPATOCELLULAR CARCINOMA

1.2.1. Imaging-Based Diagnostic Methods

Current imaging modalities for liver cancer diagnosis, including ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI), have several limitations: Difficulty in detecting small tumors at early stages; Inability to determine the biological characteristics or malignancy grade of the lesions; High cost and limited accessibility in resource-constrained settings; Risk of radiation exposure and potential adverse reactions to contrast agents; Requirement for advanced equipment and specialized technical expertise.

1.2.2. Histopathological, Cytological, and Liquid Biopsy Methods

Biopsy, involving the collection of tissue or cellular samples, is an invasive procedure that may cause pain, hemorrhage, sepsis, infection, or injury to adjacent structures. Improper localization or insufficient sample volume may lead to inaccurate or misleading diagnostic conclusions. Histopathological and cytological analyses are time-consuming, often delaying initiation of treatment. For very small or deep-seated lesions, obtaining representative samples can be technically challenging or even unfeasible. Moreover, patients with poor general health, coagulopathy, or anatomically inaccessible tumors are often unsuitable candidates for biopsy procedures.

1.2.3. Biomarker-Based Diagnostic Methods for Hepatocellular Carcinoma

1.2.3.1. Alpha –fetoprotein (AFP)

Alpha-fetoprotein (AFP) is a 70 kDa glycoprotein secreted by the fetal liver and yolk sac, which markedly declines after birth and remains at very low levels in healthy adults. The AFP gene, located on chromosome 4 (4q11–q13), is regulated by enhancer/silencer sequences; reactivation of this gene in HCC leads to elevated serum AFP levels. AFP exists in three glycoforms, among

which AFP-L3 is most strongly associated with hepatocellular carcinoma. Beyond its role as a biomarker, AFP also contributes to HCC pathogenesis by modulating innate and adaptive immune suppression, particularly through effects on macrophages, T cells, and NK cells. AFP is used for diagnosis, monitoring, and prognosis of HCC; however, its sensitivity and specificity are limited, especially for early-stage disease. A cut-off value above 200 ng/mL is generally accepted for diagnosis when supported by imaging evidence. The diagnostic performance of AFP varies by geographical region and host immunogenetic background. To enhance accuracy, AFP is frequently combined with AFP-L3 and DCP in a triple-marker model, which significantly improves screening efficiency—particularly in Asian countries with a high HCC prevalence.

1.2.3.2. DCP (des-gamma-carboxy prothrombin)

Des-gamma-carboxy prothrombin (DCP), also known as PIVKA-II (Protein Induced by Vitamin K Absence or Antagonist-II), is an abnormal prothrombin produced by hepatocytes under vitamin K deficiency. It was first reported to be elevated in HCC patients in 1984. DCP serves not only as a valuable biomarker for HCC diagnosis but also plays an active role in tumor growth and metastasis. It exhibits high sensitivity (87%) and specificity (85%) at a diagnostic threshold of 25 ng/ml, with minimal interference from benign liver diseases—thus reducing false-positive rates compared to AFP. Serum DCP levels correlate strongly with tumor size, vascular invasion, and risk of post-treatment recurrence. Importantly, DCP can detect portal vein thrombosis, a critical prognostic factor for survival. DCP also performs well in identifying early-stage HCC, including AFP-negative cases. When combined with AFP and AFP-L3, DCP yields markedly improved diagnostic accuracy, achieving an AUC of up to 0.95. Multinational and Asian studies have validated DCP as a superior serum biomarker, particularly for HBV-related HCC. Owing to its

strong diagnostic performance, DCP has been incorporated into the standard triple biomarker panel for modern HCC screening.

1.2.3.3. TXN (Thioredoxin)

Thioredoxin (TXN) is a 12 kDa protein that plays a central role in cellular redox homeostasis, DNA synthesis, and signal transduction. Together with thioredoxin reductase (TXNRD) and NADPH, it forms a protective system that maintains redox balance by reducing disulfide bonds in target proteins. TXN regulates transcription factors such as NF- κ B, HIF-1 α , and AP-1, and can also be secreted extracellularly, acting as an immunomodulatory cytokine. In cancer, TXN is often overexpressed, promoting cell proliferation, anti-apoptotic mechanisms, angiogenesis, and metastasis. In HCC, TXN activates HIF-1 α /VEGF-A and AKT/mTOR signaling pathways, interacts with BACH1, and enhances stemness and invasive potential of tumor cells. Studies have shown that serum TXN levels are significantly elevated in HCC patients and can effectively distinguish them from individuals with cirrhosis or healthy controls. When combined with AFP, TXN substantially improves diagnostic sensitivity and specificity, achieving AUC values greater than 0.97. TXN demonstrates particular efficacy in early-stage HCC detection and in AFP-negative patients. Thus, TXN represents a promising biomarker that should be integrated with AFP and DCP for comprehensive HCC screening and monitoring.

1.2.3.4. Advantages of the Combined Use of AFP, DCP, and TXN in HCC Diagnosis

Enhanced diagnostic accuracy and reliability: The combined use of three biomarkers increases diagnostic precision, particularly since each marker exhibits distinct biological properties and diagnostic performance. Comprehensive reflection of tumor heterogeneity: HCC displays diverse molecular profiles; using multiple biomarkers allows for a more accurate representation of disease status. Reduction of diagnostic errors and improved

consistency: Integrating multiple markers minimizes false results and enhances reliability across patient populations.

1.3. MONOCLONAL ANTIBODIES AND THEIR APPLICATIONS IN CANCER DIAGNOSIS

1.3.1. Concept and Representative Studies on Monoclonal Antibodies in Cancer Diagnosis

Monoclonal antibodies (mAbs) have become essential tools in cancer diagnostics owing to their high specificity for tumor-associated biomarkers. They are widely applied in detecting HER2 (breast cancer), GPC3 (hepatocellular carcinoma), and EPCA-2 (prostate cancer), improving diagnostic accuracy and facilitating early detection. mAbs are routinely used in ELISA, IHC, and flow cytometry to analyze serum, tissue, and cellular samples, and when conjugated with radioactive or fluorescent tracers, they enhance tumor visualization in PET, MRI, and SPECT imaging. The integration of nanotechnology further increases sensitivity and resolution. Beyond diagnostics, mAbs underpin personalized oncology, linking precise disease identification with targeted therapy. Their continuous refinement establishes them as a cornerstone of modern cancer diagnosis and treatment.

1.3.2. Methods for Monoclonal Antibody Production

1.3.2.1. Hybridoma Technology

The production of monoclonal antibodies via hybridoma technology is based on generating hybrid cells by fusing antigen-specific plasma cells with immortal myeloma cells. Typically, murine or rabbit B lymphocytes are used as the antibody-producing partners. The resulting hybridoma cells possess both the immortality of myeloma cells and the antibody-secreting capacity of plasma cells. These hybridomas can be cultured indefinitely, proliferating continuously and secreting large quantities of monospecific antibodies against the immunizing antigen. Importantly, hybridomas maintain long-term stability

and clonal fidelity over multiple generations, ensuring consistent antibody production for research, diagnostic, and therapeutic purposes.

1.3.2.2. Recombinant Antibody Engineering and Emerging Monoclonal Antibody Technologies

Recombinant antibody technology allows cloning of the variable heavy (VH) and light (VL) gene regions to produce antibodies in multiple formats, including scFv, Fab, and full-length IgG. These antibodies can be efficiently expressed in *E. coli*, HEK293, or CHO cells for scalable and consistent production. Humanization reduces immunogenicity and extends antibody half-life, enhancing clinical use. Alongside hybridoma methods, advanced platforms such as phage display, single B-cell culture, and single-cell transcriptomics have transformed antibody discovery, increasing diversity and precision. Collectively, these innovations underpin modern diagnostics, targeted therapy, and immunotherapy, establishing recombinant and engineered antibodies as essential tools in contemporary biomedicine.

Chapter 2. MATERIALS AND METHODS

2.1. STUDY SUBJECTS

2.1.1. Subjects for the Objective of Producing Recombinant Antigens of Three Biomarkers (AFP, DCP, and TXN)

The amino acid sequences of three proteins—Alpha-fetoprotein (AFP), Des-gamma-carboxy prothrombin (DCP), and Thioredoxin (TXN)—were retrieved from the NCBI database and used for epitope prediction analysis.

2.1.2. Subjects for the Objective of Producing Monoclonal Antibodies from the Generated Recombinant Antigens

- The purified recombinant antigens (AFP, DCP, TXN) were used to immunize experimental animals.

- Healthy, 5-week-old female BALB/c mice were selected for immunization.

2.1.3. Subjects for the Preliminary Application of Generated Monoclonal Antibodies in Early Detection of Hepatocellular Carcinoma

- Blood samples were collected from 47 patients diagnosed with hepatocellular carcinoma at various stages (early, intermediate, advanced, and late), who had not undergone surgery or received anti-cancer treatment, and had no malignancies in other organs. Samples were obtained at 108 Military Central Hospital from June 2023 to June 2024.
- Control samples included 15 healthy individuals without liver or other cancers, and 10 patients with benign liver diseases (e.g., chronic HBV, HCV infection, or cirrhosis) but without HCC, also collected at 108 Military Central Hospital during the same period.
- The study was approved by the Ethics Committee, Decision No. 06-2020/NCHG-HĐĐĐ, dated October 12, 2020.

2.2. REAGENTS, INSTRUMENTS, AND EQUIPMENT

All reagents, instruments, and laboratory equipment used in this study were provided by the Institute of Biology, Vietnam Academy of Science and Technology.

2.3. RESEARCH METHODS

2.3.1. *In Silico* Prediction of Epitope-Rich Gene Segments

Bioinformatics tools were employed to predict epitope-rich peptide regions from AFP, DCP, and TXN proteins. BepiPred 2.0 was used to predict linear epitopes (threshold ≥ 0.5). Discotope 2.0 was used to predict conformational epitopes (threshold ≥ -3.7). Antigenicity was evaluated using VaxiJen and AntigenPro, while physicochemical parameters were analyzed using ExPASy ProtParam. elected peptide sequences were then reverse-translated into nucleotide sequences using MEGA X, and NdeI/XhoI restriction sites were added for cloning.

2.3.2. Recombinant Antigen Production

The target genes (AFP, DCP, TXN) were cloned into the pET22b+ vector using restriction enzyme digestion and T4 ligase ligation, then transformed into *E. coli* BL21 (DE3) via the heat-shock method. Protein expression was

optimized by varying IPTG concentration, temperature, and induction time, and analyzed using SDS-PAGE. Recombinant proteins were purified by Ni-NTA affinity chromatography exploiting the 6×His tag, with yield estimated based on elution fractions. Protein identity was verified by Western blotting using specific monoclonal antibodies and an HRP–TMB detection system. Protein concentration was quantified via the Bradford assay, using a BSA standard curve and measuring absorbance at 595 nm with a UV–VIS spectrophotometer.

2.3.3. Monoclonal Antibody Production

BALB/c mice were immunized with the recombinant AFP, DCP, and TXN antigens emulsified with Freund's Complete/Incomplete Adjuvant (FCA/FIA) according to a 0–3–6-day immunization schedule. Following the final boost, splenic and inguinal lymphocytes were harvested and fused with myeloma cells using 50% PEG. Hybrid cells were selected in HAT medium to generate hybridomas. Hybridoma supernatants were screened via indirect ELISA to identify specific antibody-secreting clones. Positive clones were subcloned and evaluated for specificity based on the signal-to-noise ratio (SNR). Clones yielding high titers and no cross-reactivity with *E. coli* BL21 were selected for scale-up, cryopreservation, and ascites production in mice. The ascitic fluid containing antibodies was harvested, diluted, and analyzed by ELISA (OD_{450nm}). Antibody titer, SD, and CV were calculated to assess yield and preliminary neutralizing potential.

2.3.4. Purification of Monoclonal Antibodies

Antibodies were purified using affinity chromatography on Protein A/G columns, which bind to the Fc region of IgG. Hybridoma culture supernatants were centrifuged, filtered (0.22 µm), and diluted with binding buffer before being applied to the Protein A/G column. Sequential washing and elution steps were carried out using Binding Buffer and Elution Buffer, followed by

immediate neutralization of the eluate. Purified antibodies were stored at 4°C (short-term) or -20°C (long-term) to preserve activity and purity.

2.3.5. Application of Monoclonal Antibodies in ELISA-Based Detection of Antigens in Patient Serum Samples

The study evaluated the immunoreactivity of monoclonal antibodies against AFP, DCP, and TXN in serum samples from three groups: Patients with HCC, Patients with chronic liver disease without HCC, and Healthy individuals. Serum samples were prepared under standardized conditions from specimens collected at 108 Military Central Hospital. The indirect ELISA protocol was optimized for each antibody-antigen pair and performed in triplicate for reproducibility. The cut-off value was determined as $X + 3SD$ from independent negative controls. The standard curve was generated using a four-parameter logistic (4PL) model, analyzed in Python. The area under the curve (AUC) was calculated to assess the overall diagnostic sensitivity of each monoclonal antibody in detecting HCC.

2.4. DATA ANALYSIS AND STATISTICAL PROCESSING

- Data were analyzed using SPSS 2.0 and GraphPad Prism 9.0.0. Statistical methods included Chi-square (χ^2) tests, percentage analysis, mean comparison, and Kappa (K) coefficient for agreement analysis.
- Standard curves were constructed using Python.

2.5. RESEARCH SITES

- Microbial Genomics Laboratory, Institute of Biology, Vietnam Academy of Science and Technology.
- Clinical sample collection sites: Outpatient Department and A3B Department (Hepato-Biliary-Pancreatic Internal Medicine), 108 Military Central Hospital.

Chapter 3. RESEARCH RESULTS

3.1. PRODUCTION OF RECOMBINANT ANTIGENS CORRESPONDING TO THE THREE BIOMARKERS AFP, DCP, AND TXN

3.1.1. Epitope Prediction Results for AFP, DCP, and TXN Proteins

3.1.1.1. Prediction of Linear Epitopes

Based on BepiPred 2.0 analysis, AFP (609 amino acids) exhibited 21 potential epitopic regions, among which E11–E14 scored ≥ 0.62 and were prioritized for selection due to their favorable length and antigenicity score. DCP (622 amino acids) contained 16 epitopic regions, with epitope E7 (aa 281–361) showing the highest score (0.63) and occupying a structurally stable central region advantageous for antigen expression. TXN (105 amino acids) possessed two qualified epitopes (E1 and E2) with scores > 0.65 , located near the N- and central domains and enriched in charged residues, facilitating antibody recognition. These results guided the selection of epitope-rich segments for recombinant antigen design and subsequent monoclonal antibody production.

3.1.1.2. Prediction of Conformational Epitopes

AFP was modeled using Phyre2, referencing human serum albumin (PDB ID: 1e7bA) as a structural template. Discotope 2.0 identified 12 amino acids distributed in two regions (321–340 and 387–391) as potential conformational epitopes with antibody-binding capacity.

For DCP, the crystal structure 6BJR was used, revealing 70 amino acids exceeding the -3.7 threshold, forming five discontinuous epitope clusters that overlapped with previously predicted linear epitopes—thus reinforcing prediction reliability. In contrast, TXN (modeled with PDB 1ERT) contained no residues surpassing the -3.7 cutoff, indicating the absence of credible conformational epitopes, consistent with the protein's small size and compact tertiary folding. Consequently, TXN was prioritized for linear-epitope-based antigen development.

3.1.1.3. Selection of Epitope-Rich Peptide Segments

Three epitope-dense peptide regions were selected for recombinant protein expression: AFP segment (aa 319–426; 108 aa): encompasses both continuous and discontinuous epitope clusters, advantageous for immunogen design. DCP segment (aa 281–361; 81 aa): the longest continuous epitope with a high BepiPred score, containing multiple strong immunogenic regions. TXN segment (aa 5–99; 95 aa): includes both major epitopes within a structurally stable domain suitable for recombinant expression and ELISA applications.

3.1.1.4. Evaluation of Antigenicity of Selected Protein Segments

Antigenicity of P-AFP, P-DCP, and P-TXN was assessed using VaxiJen and AntigenPro with standard thresholds of 0.4–0.5. P-TXN yielded the highest scores in both tools, confirming it as a strong immunogen ideal for ELISA development and antibody generation. P-AFP showed high scores in AntigenPro, indicating good immunogenic potential despite a lower VaxiJen value. P-DCP achieved intermediate scores but contained overlapping continuous and discontinuous epitopes, enhancing its immunogenic efficiency as a recombinant antigen.

3.1.1.5. Evaluation of Physicochemical Properties of the Three Protein Segments

Physicochemical analysis using ExPASy ProtParam showed P-DCP had the highest stability and half-life but required solubility improvement, while P-TXN exhibited the best overall balance and was optimal for antigen development. P-AFP was less stable but rich in epitopes, suitable with optimized expression.

Epitope-prediction reliability: The integrated use of BepiPred 2.0, Discotope 2.0, and 3D modeling provided consistent and accurate antigenic mapping. Overlapping epitopes in AFP and DCP improved confidence, and VaxiJen/AntigenPro confirmed strong antigenicity, especially for P-TXN.

Combined with ExPASy validation, all peptides were reliable for recombinant antigen production.

3.1.1.6. Design and Optimization of Nucleotide Sequences for Recombinant Expression

The nucleotide sequences corresponding to the three peptide regions—P-AFP (348 bp), P-DCP (267 bp), and P-TXN (309 bp)—were designed using MEGA X, following standard codon usage derived from amino-acid sequences. Each construct incorporated NdeI (5' end) and XhoI (3' end) restriction sites to facilitate cloning and expression. The optimized sequences were verified to maintain open reading frames and compatibility with *E. coli* expression conditions.

3.1.2. Transformation Results in E. coli

Target genes—AFP (348 bp), DCP (267 bp), and TXN (309 bp)—were excised from the pJET1.2 vector using NdeI and XhoI, then ligated into the pET22b+ expression vector with T4 DNA ligase. The recombinant constructs were transformed into *E. coli* BL21 (DE3) by heat-shock. Ampicillin-resistant colonies were screened, and recombinant plasmids were analyzed by agarose-gel electrophoresis. Clear supercoiled DNA bands were observed, and colony PCR confirmed that insert sizes matched the designed gene lengths. These results verified the successful insertion of target genes into the expression vector, rendering the constructs ready for subsequent recombinant protein expression.

3.1.3. DNA Sequencing of AFP, DCP, and TXN Constructs

Positive *E. coli* colonies carrying plasmids containing the AFP, DCP, and TXN genes were verified by Sanger sequencing using the T7 promoter primer. The obtained sequences were aligned using ClustalW (BioEdit), showing complete identity with the original design, with no nucleotide mismatches or frame-shift errors detected. The coding regions, start codons, and T7 promoter sequences were confirmed to be correct, ensuring accurate

transcriptional activity. The verified recombinant clones were preserved for subsequent protein expression and purification.

3.1.4. Expression of Recombinant Proteins

3.1.4.1. Expression of Total Recombinant Proteins

Representative colonies harboring the AFP, DCP, and TXN genes were induced with IPTG and analyzed by SDS-PAGE. The electrophoretic profiles revealed distinct recombinant protein bands at 15 kDa (AFP), 10 kDa (DCP), and 12 kDa (TXN), matching the predicted molecular weights. These bands were absent in the control samples, confirming successful recombinant expression of all three target proteins.

3.1.4.2. Optimization of AFP, DCP, and TXN Expression Conditions

Protein expression was optimized by adjusting IPTG concentration, induction time, and temperature. Optimal conditions were AFP: 0.1 mM IPTG, 12 h, 37°C; DCP: 0.1 mM, 9 h, 25°C; TXN: 0.2 mM, 12 h, 37°C. Lower temperature favored proper DCP folding, while AFP and TXN expressed better at higher temperatures. These conditions maximized yield and minimized misfolding or inclusion body formation.

3.1.5. Purification of Recombinant Proteins

Densitometric analysis showed purification purities of 80–86% for AFP, 78–85% for DCP, and 85–90% for TXN, with overall recoveries of 73%, 71–76%, and 93–96%, respectively. TXN achieved the highest yield and purity. These results confirm efficient purification and high-quality antigen preparation suitable for further applications.

3.1.6. Validation of Purified Proteins by Western Blot Analysis

Western blotting confirmed the presence of single, well-defined bands corresponding to AFP (15 kDa), DCP (10 kDa), and TXN (12 kDa), matching the expected molecular weights. The results demonstrated high purity and structural integrity of the recombinant proteins, with no signs of degradation or aggregation. Notably, TXN exhibited outstanding stability, as

no secondary or degraded bands were observed—consistent with prior reports describing its robust structural conformation. These findings confirm that the recombinant proteins retained their specific antigenic epitopes as designed.

3.1.7. Quantification of Recombinant Protein Concentrations

Protein concentrations measured by the Bradford assay were AFP: 0.663 mg/mL, DCP: 0.328 mg/mL, and TXN: 0.851 mg/mL ($R^2 > 0.98$). Although yields were slightly lower than reported elsewhere, omitting urea minimized cytotoxicity and ensured biosafety. Combined ultrasonic and lysozyme extraction provided adequate protein quantities for immunization and diagnostic use, despite incomplete large-scale optimization

3.2. GENERATION OF MONOCLONAL ANTIBODIES AGAINST THE THREE BIOMARKERS AFP, DCP, AND TXN FROM RECOMBINANT ANTIGENS

3.2.1. Immunization of BALB/c Mice

BALB/c mice immunized with AFP, DCP, and TXN recombinant antigens (400 $\mu\text{g/mL}$, emulsified with FCA/FIA) remained healthy, with normal behavior and weight. All immunized mice exhibited strong immune responses consistent with effective antibody induction. The immunization design (FCA prime, FIA boosters) and antigen dose (20 μg per injection) followed international standards, ensuring biosafety and suitability for monoclonal antibody production in BALB/c mice.

3.2.2. Fusion of Mouse Lymphocytes and Myeloma Cells

3.2.2.1. Recovery and maintenance of myeloma cells

Myeloma cells were thawed, cultured under 5% CO_2 , and showed normal morphology and proliferation within 48 h. By 72 h, 80% confluence was achieved, confirming healthy recovery and sufficient cell density for fusion.

3.2.2.2. Fusion results

After the second fusion, hybrid cells were cultured in HAT medium on 96-well plates. Hybrid colonies appeared in ~30% of wells by day 5 and in 70% by day 10, with 80% surface coverage—indicating successful fusion. These kinetics aligned with reported optimal hybridoma formation (7–10 days), suitable for subsequent ELISA screening.

3.2.3. Screening and Cloning of Antibody-Secreting Hybridomas

ELISA screening validated the assay system with clear positive/negative controls and high specificity. Six hybridoma clones—AFP1/2, DCP1/2, and TXN1/2—produced strong specific signals ($\text{SNR} \geq 3.64$) against recombinant antigens without cross-reactivity to *E. coli* BL21. These clones were expanded for ascites induction and further antibody development.

3.2.4. Ascites Production and Antibody Harvesting

3.2.4.1. AFP1/AFP2:

AFP1 induced ascites in 4/5 mice within 6–7 days (11 mL total, ≈ 3 mL/mouse). AFP2 achieved 100% yield (5/5 mice) after 9–10 days, totaling 12 mL—demonstrating efficient antibody production.

3.2.4.2. DCP1/DCP2

DCP1 produced ascites in 4/5 mice within 5–6 days (12 mL total), while DCP2 achieved full success (5/5 mice, 14 mL total). Both lines showed high hybridoma stability and strong antibody yield..

3.2.4.3. TXN1/TXN2

Both lines induced ascites in all mice (5/5). TXN1 yielded 11 mL after 6 days; TXN2, 13 mL after similar dosing. Consistent yields confirmed high ascites efficiency.

3.2.4.4. Overall evaluation

Ascites formation occurred within 5–10 days—significantly faster than typical protocols—indicating an optimized process. Variations in yield

among lines (e.g., AFP1, DCP1 at ~80%) likely reflected clone-specific growth or host immune variation.

3.2.5. Antibody Titer Evaluation in Ascitic Fluids

3.2.5.1. AFP1/AFP2

Both clones produced high-titer antibodies ($OD_{450} > 2.8$ at 1:100–1:200 dilutions; low CV%), with AFP2 maintaining stronger reactivity at higher dilutions—indicating superior immunoreactivity.

3.2.5.2. DCP1/DCP2

Both generated specific anti-DCP antibodies with stable curves and $CV < 10\%$. DCP1 exhibited consistently higher titers than DCP2.

3.2.5.3. TXN1/TXN2

Both produced specific anti-TXN antibodies; TXN1 had higher titers across dilutions, while TXN2 showed better stability at lower dilutions.

3.2.5.4. Overall comparison

AFP1, AFP2, and DCP1 displayed the highest titers ($OD_{450} > 2.8$ at 1:200; > 1.7 at 1:400–800). DCP2, TXN1, and TXN2 also met the required thresholds ($OD_{450} \approx 1.2$ at 1:400). All six lines thus qualified as high-affinity monoclonal antibody producers.

3.2.6. Purification of Monoclonal Antibodies

Purified monoclonal antibodies against AFP, DCP, and TXN displayed the characteristic IgG heavy (50 kDa) and light (25 kDa) chains on SDS-PAGE, with no extraneous bands. The Protein G affinity purification yielded products of $>95\%$ purity, confirming the high efficiency and integrity of the purification process.

3.3. PRELIMINARY APPLICATION OF MONOCLONAL ANTIBODIES IN ELISA FOR THE DETECTION OF HCC

3.3.1. General Characteristics of Study Groups

3.3.1.1. HCC Patients

Among 47 HCC patients, those aged ≥ 60 years accounted for 72.34%, consistent with global trends, though cases also occurred in individuals < 45 years. Males predominated (87.2%; ratio 7:1), reflecting gender-related differences in lifestyle, hormones, and immunity. HBV infection was the leading cause (81%), followed by HCV (10.6%) and alcoholic cirrhosis (6.3%). Only 17% were diagnosed at early stages, underscoring major challenges in early detection.

3.3.1.2. Non-HCC Groups

The non-HCC cohort included 10 patients with liver diseases (cirrhosis, chronic HBV, liver abscess, HBV/HCV coinfection) and 15 healthy subjects aged 38–79. No HCC cases were found among those with chronic liver disease, indicating HCC can occur even without cirrhosis, particularly in chronic HBV infection.

3.3.1.3. Liver Function Biochemistry

Liver biochemical markers differed significantly across groups ($p < 0.001$). The non-cancer liver disease group had the highest AST, ALT, bilirubin, and PT-INR and the lowest albumin, indicating severe hepatic dysfunction. The HCC group showed moderate enzyme elevation and preserved albumin, suggesting compensated cirrhosis.

3.3.2. ELISA Assay Results

3.3.2.1. Optimization of Indirect ELISA Conditions

Optimal binding occurred at antibody dilutions 1:64–1:128 and antigen dilutions 1:8–1:16, yielding peak OD₄₅₀ values of 1.849 (AFP), 1.865 (DCP), and 1.975 (TXN). Serum dilution 1:5, 1.5% BSA blocking, and HRP-conjugated secondary antibody at 1:15,000 provided the highest signal-to-noise ratios ($P/N > 5$). TXN showed the greatest sensitivity. These optimized conditions ensured strong signals, low background, and high specificity for quantitative analysis.

3.3.2.2. Construction of 4-Parameter Logistic Standard Curves

All standard curves fit the 4PL model well ($R^2 > 0.99$).

AFP: $EC_{50} = 8694.2$ ng/mL, slope -1.30 , stable baseline, suitable for low-level detection. DCP: $EC_{50} = 222.754$ ng/mL, lower affinity but robust at higher antigen levels. TXN: $EC_{50} = 1687.9$ ng/mL, highest OD_{max} (2.02), optimal for AFP-negative cases. All antibodies exhibited high sensitivity and specificity, and the AFP–DCP–TXN panel showed excellent linearity and diagnostic potential for multi-marker ELISA development.

3.3.2.3. Sample Analysis Using Cut-off Thresholds

Positive rates ranged 50–65% depending on the cut-off. AFP showed the highest rate but limited discrimination; DCP performed similarly but improved when combined with AFP. TXN provided clearer separation and stronger OD signals. The combined use of AFP + DCP + TXN enhanced both sensitivity and specificity, suggesting strong potential for multi-marker ELISA kits, especially for early HCC detection in chronic hepatitis.

3.3.2.4. Sensitivity and Specificity Compared with Clinical Diagnosis

Analysis of 72 serum samples showed: AFP: Se 89.36%, Sp 68%, Kappa = 0.59; DCP: Se 91.48%, Sp 80%, Kappa = 0.72; TXN: Se 95.74%, Sp 84%, Kappa = 0.813

Combined AFP+DCP+TXN yielded Se 97.87%, Sp 92%, PPV/NPV 95.83%, Kappa = 0.907, nearly perfect agreement with clinical diagnosis. This confirms the superiority of multi-marker detection and highlights TXN's utility in AFP-negative HCC.

3.3.2.5. Comparison with Commercial Antibodies

The in-house monoclonal antibodies showed high concordance with commercial kits: AFP (95.83%), DCP (94.44%), TXN (93.05%), with Kappa values 0.903, 0.878, and 0.842, respectively—indicating near-perfect agreement. Minor discrepancies were attributed to cross-reactivity or patient

variability. TXN specificity can be further enhanced by purification or multiplex/electrochemiluminescent approaches.

3.3.2.6. ROC Curve Analysis

The AUC values for AFP, DCP, and TXN were 0.719, 0.761, and 0.774, respectively, indicating moderate to good classification ability. Combining all three markers increased the AUC to 0.87, confirming superior performance over individual assays. These results demonstrate that multi-marker ELISA offers a reliable and sensitive method for early HCC screening.

CONCLUSION

1. Production of recombinant antigens AFP, DCP, and TXN

The study selected three peptide fragments containing continuous and discontinuous epitopes of three biomarkers, AFP, DCP, and TXN, based on criteria of antigenicity, solubility, stability, and expression. The corresponding encoding genes were synthesized and transferred into the pET22b+ vector. When expressed in *E. coli* BL21, they yielded recombinant proteins of the correct size (10–15 kDa), purified by Ni-NTA affinity chromatography with purity ranging from 78% to 90%. Western blot results showed that the obtained antigens had suitable structure and antigenicity, ensuring immunological value for subsequent steps. The results confirm the novelty of the thesis, as it is the first time in Vietnam that three recombinant antigens, AFP, DCP, and TXN, with high antigenicity have been created, serving as specific materials for the production of monoclonal antibodies for the diagnosis of liver cancer.

2. Production of specific monoclonal antibodies from recombinant antigens

Based on the recombinant antigens created, six hybridoma cell lines (AFP1, AFP2, DCP1, DCP2, TXN1, TXN2) were generated by fusing mouse B lymphocytes with myeloma cells. These cell lines developed stably and produced corresponding monoclonal IgG antibodies with high titers obtained

in mouse ascites fluid BALB/c (approximately 11–14 ml per mouse sample). The obtained antibodies were purified by Protein A/G chromatography. Under SDS-PAGE gel, the two corresponding heavy and light chains of the antibody were clearly visible, confirming that the antibodies were purified and achieved high purity (>95%). Thus, the study has created six new hybridoma lines secreting monoclonal antibodies specific to the recombinant antigens AFP, DCP, and TXN, which have never been published domestically, with the potential for direct application in immunological and ELISA diagnostic tests.

3. Application of monoclonal antibodies for HCC detection

The generated monoclonal antibodies were employed in indirect ELISA to detect AFP, DCP, and TXN in patient sera. After optimization and 4-parameter logistic curve fitting, individual assay performance showed: AFP: Sensitivity 89.36%, Specificity 68.00%; DCP: Sensitivity 91.48%, Specificity 80.00%; TXN: Sensitivity 95.74%, Specificity 84.00%; When combining $\geq 2/3$ markers, overall sensitivity 97.87%, specificity 92.00%, and PPV/NPV = 95.83% were achieved. Concordance with commercial kits exceeded 93%, confirming reliability and diagnostic equivalence. This marks the first successful development in Vietnam of a recombinant monoclonal antibody trio (AFP–DCP–TXN) for ELISA-based HCC diagnosis, significantly improving detection accuracy, particularly in AFP-negative cases, and paving the way toward localized production of cancer diagnostic reagents.

4. Limitations

- Limited sample size (47 HCC and 10 non-malignant liver cases) restricts full sensitivity and specificity assessment across liver pathologies.
- The ELISA protocol remains manual and laboratory-scale, requiring adaptation for routine clinical testing.
- Antibody production is still small-scale, with stability and reproducibility at larger production volumes yet to be evaluated.

RECOMMENDATIONS

1. **Clinical validation:** Conduct large-scale, multicenter studies to confirm clinical reliability and real-world performance of the AFP, DCP, and TXN monoclonal antibody panel, especially for early-stage HCC where AFP alone is often negative. Expanded trials across hospitals will help assess specificity and diagnostic discrimination.
2. **Technical standardization and pilot implementation:** Standardize the ELISA workflow using in-house antibodies, including stability, storage, and operating protocols. Implement pilot testing of small-scale ELISA kits at provincial hospitals to evaluate field stability and user feedback. Results will form the scientific and regulatory basis for commercial registration of a Vietnam-made diagnostic kit

LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

1. **Ho Truong Giang**, Vo Thi Bich Thuy, Nghiem Ngoc Minh (2024), Development of hybridoma cell lines producing monoclonal antibodies against recombinant alpha-fetoprotein antigen. *Vietnam Medical Journal*, 539(Special Issue, June), 437–446..
2. Vo Thi Bich Thuy, **Ho Truong Giang**, Nguyen Thi Diem, Tran Thi Hop, Nghiem Ngoc Minh (2024), Generation of hybridoma cell lines producing monoclonal antibodies against recombinant des-gamma-carboxy prothrombin antigen. *Thai Nguyen University Journal of Science and Technology*, 229(13), 394–400.
3. Huy Quang Duong, **Ho Truong Giang**, Nguyen Thi Diem, Thuy Thi Bich Vo (2025), Evaluation of the sensitivity and specificity of monoclonal antibodies for detecting the thioredoxin antigen in hepatocellular carcinoma patients. *Edelweiss Applied Science and Technology*, 2025, Vol. 9, No. 1: 1010-1018.