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CANCER PROGNOSIS

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Meet the editor

Guy-Joseph Lemamy, PhD, is currently a professor in the Department of Cellular and Molecular Biology-Genetics, and Director of the Laboratory of Cellular and Molecular Biopathologies, at the Faculty of Medicine, Université des Sciences de la Santé (USS), Libreville, Gabon. Dr. Lemamy obtained his PhD in Biochemistry and Cell Biology at the Faculty of Medicine, Université Montpellier 1, France. His PhD thesis work concerned the search for new tumor markers in breast cancers, led by the Medical Research Institute (INSERM U148) in Montpellier, France. Dr. Lemamy is the author of many book chapters and journal articles about tumor markers and is involved in other scientific activities, such as membership of the Scientific Advisory Board of Gabon Scientific Research Guiding Plan.

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Introduction

Chapter 1

Introductory Chapter: Genes Expression in the Control of Cell Cycle and Their Potential Value in Cancer Prognosis

Guy Joseph Lemamy

Additional information is available at the end of the chapter

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1. Introduction

The human body is made of up to trillions of cells. Those cells respond to three characteristics: the differentiation into up to 200 different cellular types which acquire specific functions, the cooperation between those cells using various signaling pathways to sustain the body's physiological unity, and the genetic programming of cell death (the apoptosis). The programmed cell death goes hand in hand with the sustainability of life highlighted at the cell level by the "immortality" of cancer cells, the permanent renewal process of certain tissues such as those of the intestine and blood, as well as the sustainability of the species.

The cancer is a genomic disease in which the early stage is represented by activation of oncogene and inactivation of suppressor genes, which result in transformed cells that grow out of cell cycle control. Two families of genes, the oncogenes and antioncogenes (also called tumor suppressor genes), cause and accelerate the carcinogenesis process when their structure or the regulation of their expression is altered. These genes are equivalent in cancer etiology but differ themselves by their functions and by the mechanisms of their activation. The expression of oncogenes and antioncogenes, respectively, controls in a positive and negative way the cell cycle progression [1] (**Figure 1**).

Among the oncogenes, HER2 is involved in the early stages of carcinogenesis. HER2 is located on chromosome 17 [2] and codes for a transmembrane tyrosine kinase receptor that belongs to a family of four members: human epidermal growth factor receptor (HER) [3]. HER is activated after the binding of its ligand which allows the phosphorylation of tyrosine residues in the intracellular domain of the receptor. This activation leads to signaling pathways promoting cell proliferation, survival, migration, adhesion, angiogenesis, or differentiation

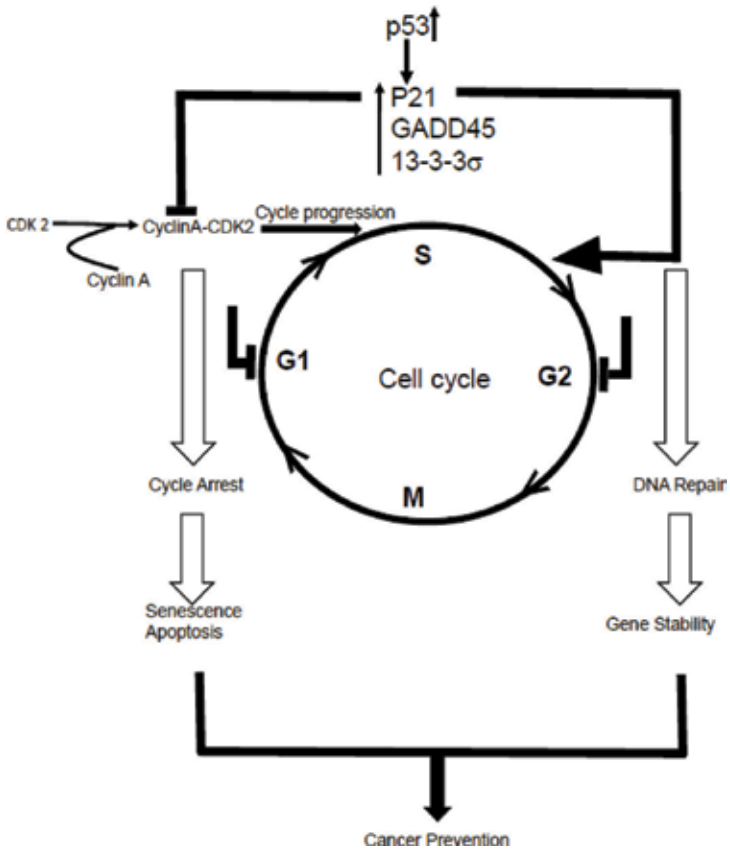

Figure 1. Signaling pathways of p53 in gene stability and cancer prevention. DNA damage increases p53 protein levels that stimulate expression of various genes such as p21, GADD45, or 13-3-3σ. The progression of cell cycle activated by cyclin-CDK complexes is temporarily inhibited by the resulting proteins at the G1 and G2 phases (⊥). Meanwhile, the exonuclease activity of p53 helps to repair damaged DNA. Cells with serious DNA mutations are directed to senescence or apoptosis.

[4]. In breast cancer cells, HER2 gene is amplified followed by the protein overexpression [5]. HER2 overexpression enhances cell proliferation through rapid degradation of the cyclin-dependent kinase (CdK) inhibitor p27 and the upregulation of factors that promote cell cycle progression such as CdK6 and cyclins D1 and E. HER2 is used as prognostic, prognosis, and predictive biomarker in breast cancer [6] (**Figure 2**).

In human cancer, the p53 tumor suppressor gene is the most commonly mutated gene. The p53 gene is mapped to the region 17p13.1, on the short arm of chromosome 17 [7], and codes for the p53 protein (tumor protein of 53 kDa) [8]. The p53 protein plays a main role in cell cycle control and cancer prevention. Damages in DNA occurring during the cell cycle or oncogene activation induce the p53 protein accumulation, resulting in temporary cell cycle arrest at the G1 and G2 checkpoints, DNA repair, differentiation, senescence, apoptosis, or antiangiogenesis. The p53 protein stimulates the expression of other genes such as p21, growth arrest and DNA damage-inducible 45 proteins (GADD45), 13-3-3σ, etc. These proteins are implicated in inactivation of cyclin-cyclin-dependent kinase (cyclin-CDK) complexes required for the progression of the cell cycle (**Figure 1**).

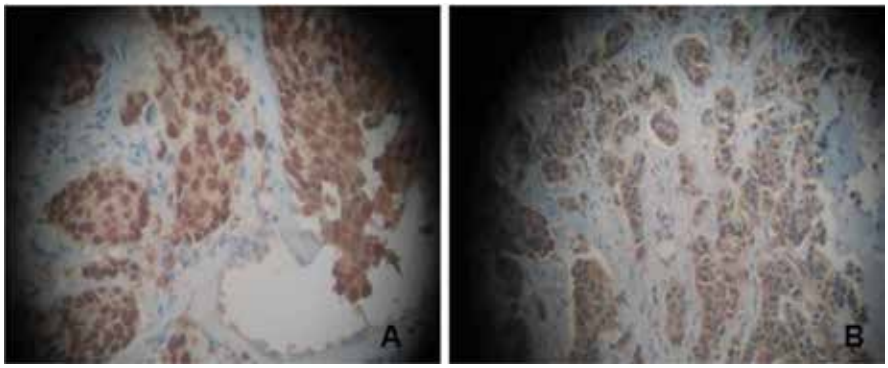

Figure 2. Immunostaining (brown) of estrogen receptor (ER) and HER2 in ductal invasive breast carcinoma. ER (A) and HER2 (B) were revealed, respectively, by their specific antibody. The tissue was counterstained by hematoxylin (blue). Magnification by light microscopy ×400 (from collaboration between Department of Surgical Pathology and Department of Cellular and Molecular Biology-Genetics; Faculty of Medicine, University of Health Sciences, Libreville, Gabon).

In breast cancer, other tumor suppressor genes such as BRCA1 or BRCA2 [9–11] code for proteins that repair DNA damages. Mutations of these genes result in mutated proteins that cannot repair DNA, and cells bearing such mutations will turn into cancers. Taken together, the mutations of BRCA1 and BRCA2 cause about 20–25% of inherited breast cancer and 5–10% of all cancers [12].

Breast cancer risk is of about 50–80% in women with a genetic predisposition [13, 14]. Furthermore, it has been shown that mutation of BRCA1 and BRCA2 is a predictive factor of a major risk of breast cancer [15].The tumor progression can be locally favored by mitogenic effects of hormones, or growth factors which stimulate the tumor's growth, or by activating vascular endothelial growth factor (VEGF) receptor to induce angiogenesis. Human breast cancer is characterized by its high sensitivity to estrogens and its high metastatic potential. About 50% of these cancers are sensitive to antiestrogen treatment [16]. Therefore, the presence of estrogen receptor (ER) is considered to be a predictive factor of response to hormone therapy. Moreover, ER has been shown to be an independent prognostic factor in mammary cancer [16] (**Figure 2**).

Furthermore, estrogens and growth factors induce proteases such as cathepsin D [17]. Cathepsin D is an acid aspartyl endoprotease that is routed through the trans-Golgi network (TGN), by binding to the mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2-R) via M6P signals, to lysosomes for degradation. In cancer cells, cathepsin D acts as mitogen promoting metastases [18, 19] and tumor angiogenesis [20, 21]. Clinical studies revealed that cathepsin D is an independent prognostic factor for metastasis risk in breast cancer [22]. In parallel, experimental studies in breast cancer cell lines showed deficiencies of cathepsin D routing to lysosomes, suggesting defects at the receptor level.

Therefore, the M6P/IGF2-R has been hypothesized as being coded by a breast cancer suppressor gene [23], and its potential prognostic significance in breast cancer has been suggested [24].

The cancer pathology includes more than 100 diseases that can cause serious illness or death if not detected in time. The purpose of this book is to present current developments in the methodology in cell and molecular biology which have deeply advanced in the understanding of cancer's prevention and prognosis. Among them, the research for biomarkers may be

essential since they can be the target of a preventive therapy, a marker of risk that can be used to identify populations with high risk or a marker of a drug's toxicity used in prevention which can help to monitor its tolerance.

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Section 2

Molecular Markers Studies

Chapter 2

The Human Epidermal Growth Factor Receptor 2 (HER2) as a Prognostic and Predictive Biomarker: Molecular Insights into HER2 Activation and Diagnostic Implications

Daniela Furrer, Claudie Paquet, Simon Jacob and Caroline Diorio

Additional information is available at the end of the chapter

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Abstract

The human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor protein. *HER2* gene amplification and receptor overexpression, which occur in 15–20% of breast cancer patients, are important markers for poor prognosis. Moreover, HER2-positive status is considered a predictive marker of response to HER2 inhibitors including trastuzumab and lapatinib. Therefore, reliable HER2 determination is essential to determine the eligibility of breast cancer patients to targeted anti-HER2 therapies. In this chapter, we aim to illustrate important aspects of the HER2 receptor as well as the molecular consequences of its aberrant constitutive activation in breast cancer. In addition, we will present the methods that can be used for the evaluation of HER2 status at different levels (protein, RNA, and DNA level) in clinical practice.

Keywords: breast neoplasm, oncogene, tyrosine kinase receptor, molecular oncology, HER2 status, HER2 inhibitors

1. Introduction

Breast cancer is the most frequently diagnosed cancer among women worldwide, affecting over 1.5 million women each year. In 2015, it is estimated that worldwide 500,000 women have died from this malignancy, which represents 15% of all cancer-related deaths among women [1].

It is now well recognized that breast cancer comprises a heterogeneous group of diseases in term of differentiation and proliferation, prognosis and treatment. Over the past decades, microarray-based gene expression studies have allowed the identification of breast cancer intrinsic subtypes [2–4]. One of these subtypes is the so-called human epidermal growth factor receptor 2 (HER2)-enriched subtype. HER2 is a transmembrane tyrosine kinase receptor [5]. This protein is encoded by the *HER2* gene, which is located on the long arm of chromosome 17 (17q12–21.32) [6]. The HER2-enriched subtype is characterized by high expression of HER2 and other genes of the 17q amplicon, including growth factor receptor bound protein 7 (GRB7), and low to intermediate expression of luminal genes such as Estrogen Receptor 1 (ESR1) and Progesterone Receptor (PGR) [7]. Clinically, HER2-positive breast cancer occurs in 15–20% of breast cancer patients and is characterized by the overexpression of the HER2 receptor and/or *HER2* gene amplification [8]. HER2-positive breast cancer patients have a particular worse prognosis. Importantly, HER2-positive breast cancer patients are eligible to receive targeted treatment with trastuzumab, a monoclonal antibody specifically directed against the HER2 receptor [9]. Trastuzumab treatment, in combination with chemotherapy, improves the outcome of early [10, 11] and metastatic [12, 13] HER2-positive breast cancer patients. The US Food and Drug Administration (FDA) approved trastuzumab for the treatment of metastatic HER2-positive breast cancer patients in 1998 and for the treatment of early HER2-positive breast cancer patients in 2006. Lapatinib is a small-molecule inhibitor of the intracellular tyrosine kinase domain of both HER2 and EGFR receptors [14]. Lapatinib has received FDA approval in 2007 as combination therapy with capecitabine for the treatment of patients with HER2-positive advanced breast cancer patients who had progressed on trastuzumab-based regimens [15]. Although anti-HER2 agents are generally well tolerated, trastuzumab administration has been associated with cardiac side effects, especially when used in combination with anthracyclines [16].

HER2 plays a significant role in breast cancer pathogenesis. It is therefore essential to understand the biology of this receptor in order to better treat HER2-positive breast cancer patients. Evaluation of HER2 status in breast cancer specimens raises several technical considerations. In the last decades, several methods have been developed for HER2 assessment. In this article, we will review important aspects of the HER2 biology and its relevance in breast cancer and present the techniques that are used in clinical practice for the determination of HER2 status in breast cancer specimens.

2. HER2 biology and methods of assessment of HER2 status

2.1. HER2 receptor

The HER2 receptor is a 185 kDa transmembrane protein that is encoded by the *HER2* (also known as *erb-b2 receptor tyrosine kinase 2 [ERBB2]*) gene, which is located on the long arm of chromosome 17 (17q12–21.32) [6]. HER2 is normally expressed on cell membranes of epithelial cells of several organs like the breast and the skin, as well as gastrointestinal, respiratory, reproductive, and urinary tract [17]. In normal breast epithelial cells, HER2 is expressed at low levels (two copies of the *HER2* gene and up to 20,000 HER2 receptors) [18], whereas in HER2-positive breast cancer cells, there is an increase in the number of *HER2* gene copies (up to 25–50, termed gene amplification) and HER2 receptors (up to 40 to 100 fold increase,

termed protein overexpression), resulting in up to 2 million receptors expressed at the tumor cell surface [19]. Besides breast cancer, HER2 overexpression has also been reported in other types of tumors, including stomach, ovary, colon, bladder, lung, uterine cervix, head and neck, and esophageal cancer as well as uterine serous endometrial carcinoma [20].

2.1.1. HER2 structure and function

HER2 belongs to the epidermal growth factor receptor (EGFR) family. This family is composed of four HER receptors: human epidermal growth factor receptor 1 (HER1) (also termed EGFR), HER2, human epidermal growth factor receptor 3 (HER3), and human epidermal growth factor receptor 4 (HER4) [5].

HER family members are transmembrane receptor tyrosine kinases. Tyrosine kinases are enzymes that carry out tyrosine phosphorylation, namely the transfer of the γ phosphate of adenosine triphosphate (ATP) to tyrosine residues on protein substrate [21].

HER receptors share a similar structure. They are composed of an extracellular domain (ECD), a transmembrane segment and an intracellular region [22]. The ECD domain is divided into four parts: domains I and III, which play a role in ligand binding, and domains II and IV, which contain several cysteine residues that are important for disulfide bond formation [23]. The transmembrane segment is composed of 19–25 amino acid residues. The intracellular region is composed of a juxtamembrane segment, a functional protein kinase domain (with the exception of HER3 that lacks tyrosine kinase activity [24] and must partner with another family member to be activated [25]), and a C-terminal tail containing multiple phosphorylation sites required for propagation of downstream signaling [23]. The catalytic domain contains the ATP binding pocket, a conserved site essential to ATP binding [26].

HER receptors are activated by both homo- and heterodimerization, generally induced by ligand binding [27]. This suggests that HER receptor family has evolved to provide a high degree of signal diversity [28]. The cellular outcome produced by HER receptors activation depends on the signaling pathways that are induced, as well as their magnitude and duration, which are influenced by the composition of the dimer and the identity of the ligand [28].

Several growth factor ligands interact with the HER receptors [29]. HER1 receptor is activated by six ligands: epidermal growth factor (EGF), epigen (EPG), transforming growth factor α (TGFα), amphiregulin, heparin-binding EGF-like growth factor, betacellulin and epiregulin. HER3 and HER4 receptors bind neuregulins (neuregulin-1, neuregulin-2, neuregulin-3, and neuregulin-4). HER2 is a co-receptor for many ligands and is often transactivated by EGF-like ligands, inducing the formation of HER1-HER2 heterodimers. Neuregulins induces the formation of HER2-HER3 and HER2-HER4 heterodimers [29]. However, no known ligand can promote HER2 homodimer formation, implying that no ligand can bind directly to HER2 [30].

The structural basis for receptor dimerization has been elucidated in recent years through crystallographic studies [31, 32]. Dimerization is mediated by the dimerization arm, a region of the extracellular region of HER receptors. While in its inactivated state the dimerization arm of EGFR, HER3 and HER4 is hidden, ligand binding induces a receptor conformational change leading to exposure of the dimerization arm [31]. In contrast to the other three HER receptors, the dimerization arm of the HER2 receptor is permanently partially exposed, thus permitting its dimerization even if the HER2 receptor lacks ligand-binding activity [32].

Interaction between the dimerization arms of two HER receptors promotes the formation of a stable receptor dimer in which the kinase regions of both receptors are closed enough to permit transphosphorylation of tyrosine residues, i.e. the transfer of a phosphate group by a protein kinase to a tyrosine residue in a different kinase molecule [33, 34]. The first member of the dimer mediates the phosphorylation of the second, and the second dimer mediates the phosphorylation of the first [23].

The phosphorylation of specific tyrosine residues following HER receptor activation and the subsequent recruitment and activation of downstream signaling proteins leads to activation of downstream signaling pathways promoting cell proliferation, survival, migration, adhesion, angiogenesis and differentiation [35]. The Phosphatidylinositol 3′-kinase (PI3K)-Akt pathway and the Ras/Raf/MEK/ERK pathway (also known as extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway) are the two most important and most extensively studied downstream signaling pathways that are activated by the HER receptors [5, 36]. These downstream signaling cascades control cell cycle, cell growth and survival, apoptosis, metabolism and angiogenesis [37, 38]. Signaling from HER receptors is then terminated through the internalization of the activated receptors from the cell surface by endocytosis. Internalized receptors are then either recycled back to the plasma membrane (HER2, HER3, HER4) or degraded in lysosomes (HER1) [39, 40].

HER heterodimers produce more potent signal transduction than homodimers. This can be explained by the fact that heterodimerization provides additional phosphotyrosine residues necessary for the recruitment of effector proteins [28]. Heterodimerization follows a strict hierarchical principle with HER2 representing the preferred dimerization and signaling partner for all other members of the HER family [41]. HER2 seems to function mainly as a co-receptor, increasing the affinity of ligand binding to dimerized receptor complexes [42, 43]. HER2 has the strongest catalytic kinase activity [41] and HER2-containing heterodimers produce intracellular signals that are significantly stronger than signals generated from other HER heterodimers [44]. The HER2-HER3 heterodimer in particular exhibits extremely potent mitogenic activity through the stimulation of the PI3K/Akt pathway, a master regulator of cell growth and survival [45]. Furthermore, HER2 containing heterodimers have a slow rate of receptor internalization, which results in prolonged stimulation of downstream signaling pathways [28]. HER2 can also be activated by complexing with other membrane receptors, such as Insulin-like growth factor I receptor (IGF-1R) [46].

2.1.2. Consequences of constitutive HER2 receptor activation

Whereas in normal cells the activity of tyrosine kinases is a tightly controlled mechanism, in cancer cells, alterations in tyrosine kinases—overexpression of receptor tyrosine kinase proteins, amplification or mutation in the corresponding gene, abnormal stimulation by autocrine growth factors loop or delayed degradation of activated receptor tyrosine kinase—lead to constitutive kinase activation and therefore to aberrant cellular growth and proliferation [34, 47]. Constitutive activation of HER1, HER2, HER3, IGF-1R, Fibroblast growth factor receptor (FGFR), c-Met, Insulin Receptor (IR), Vascular Endothelial Growth Factor Receptor (VEGFR), Jak kinases and Src have been associated with human cancer [34, 48–52].

Several ways of aberrant activation of HER receptors have been described, including ligand binding, molecular structural alterations, lack of the phosphatase activity, or overexpression of the HER receptor [53].

In HER2-positive tumors, receptor overexpression has been identified as the mechanism of HER2 activation. The increased amount of cell surface HER2 receptors associated with HER2 overexpression leads to increased receptor-receptor interactions, provoking a sustained tyrosine phosphorylation of the kinase domain and therefore constant activation of the signaling pathways. HER2 overexpression also enhances HER2 heterodimerization with HER1 and HER3 [54] resulting in an increased activation of the downstream signaling pathways. It has also been shown that HER2 overexpression leads to enhanced HER1 membrane expression and HER1 signaling activity through interference with the endocytic regulation of HER1 [54–56]. While HER1 undergoes endocytic degradation after ligand-mediated activation and homodimerization, HER1-HER2 heterodimers evade endocytic degradation in favor of the recycling pathway [57, 58], resulting in increased HER1 membrane expression and activity [55, 56, 59].

It has also been reported that HER2 overexpression enhances cell proliferation through the rapid degradation of the cyclin-dependent kinase (Cdk) inhibitor p27 and the upregulation of factors that promote cell cycle progression, including Cdk6 and cyclins D1 and E [60].

Several methods have been developed for the assessment of HER2 status in breast cancer specimens, at the protein level, DNA level, and RNA level. Here below, we present some of the existing techniques that are used for the HER2 determination in clinical practice.

2.2. Methods for the evaluation of HER2 status in breast cancer specimens

2.2.1. HER2 status evaluation at the protein level

2.2.1.1. Immunohistochemistry (IHC)

IHC allows the evaluation of the HER2 protein expression in formalin-fixed, paraffin-embedded (FFPE) tissues using specific antibodies directed against the HER2 receptor protein [61]. HER2 receptor is then visualized with the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB) resulting in a brownish membranous staining. Several commercially available diagnostic tests for the determination of HER2 expression have been approved by the FDA: the HercepTest™ kit (DAKO, Glostrup, Denmark), the InSite™ HER2/neu kit (clone CB11; BioGenex Laboratories, San Ramon, CA), the Pathway™ kit (clone 4B5; Ventana Medical Systems, Tucson, AZ), and the Bond Oracle HER2 IHC System (Leica Biosystems, Newcastle, UK).

By this method, it is possible to estimate the number of cells showing membranous staining in the tissue section as well as the intensity of the staining [62]. Membranous staining in the invasive component of specimen is scored on a semi-quantitative scale. According to the American Society of Clinical oncology (ASCO) and the College of American Pathologists (CAP) recommendations for HER2 testing in breast cancer published in 2013, HER2 expression is scored as 0 (no staining or weak/incomplete membrane staining in ≤10% of tumor cells), 1+ (weak, incomplete membrane staining in >10% of tumor cells), 2+ (strong, complete membrane

staining in ≤10% of tumor cells or weak/moderate and/or incomplete membrane staining in >10% of tumors cells) or 3+ (strong, complete, homogeneous membrane staining in >10% of tumor cells) [61]. In clinical practice, HER2 immunohistochemical status is evaluated as negative if the immunohistochemical score is 0 or 1+, equivocal is the score is 2+, and positive if the score is 3+. Patients with a positive HER2 status at the IHC are eligible for targeted therapy with HER2 inhibitors. The IHC 2+ category is considered borderline and confirmatory testing using an alternative assay (fluorescence *in situ* hybridization (FISH) or other *in situ* hybridization (ISH) methods, see Section 2.2.2) is required for final determination.

IHC is an easy and relatively inexpensive method [63]. However, this technique can be affected by numerous factors, including warm/cold ischemic time [64], delay and duration of fixation [65], and antibody used [66, 67]. Moreover, since the interpretation of results is based on semiquantitative scoring, this technique is prone to interobserver variability and therefore to substantial discrepancies in the IHC results, particularly for cases scoring 2+ [68].

2.2.1.2. Enzyme-linked immunosorbent assay (ELISA)

As mentioned before, HER2 receptor is composed of an extracellular domain (ECD), a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The HER2 ECD can be cleaved from the HER2 full-length receptor through matrix metalloproteases and released into the serum [69]. HER2 ECD levels present in serum can be measured using an enzyme-linked immunosorbent assay (ELISA). HER2 ECD is detected using two antibodies that recognize two specific epitopes of the antigen. Several commercially available ELISA assays received FDA approval: the automated ELISA assay Immuno-1 (Siemens Healthcare Diagnostics, Tarrytown, NY), the manual ELISA assay (Siemens Healthcare Diagnostics) in 2000, and the automated ELISA assay ADVIA Centaur (Siemens Healthcare Diagnostics) in 2003 [70].

Although some studies suggest that HER2 ECD levels measured in patient's serum could be used as a biomarker for the monitoring of the disease course and the response of the patient to therapy, the clinical use of the ELISA assay for the evaluation of the HER2 ECD has not yet been widely implemented [71, 72]. This is mainly due to the fact that studies that analyzed the association between HER2 ECD levels and prognostic and predictive factors in breast cancer patients reported conflicting results, depending on which cutoff value was considered or which assay was used [71].

ELISA is an easy and fast method. In addition, given that HER2 ECD can be measured directly in serum, ELISA can be used to monitor the dynamic changes of HER2 status following treatment or over the course of the disease progression [71]. Results obtained by ELISA, however, might not be reliable if the serum samples are from patients under treatment, as trastuzumab present in the patient's serum might compete with the two antibodies used in the assay.

2.2.2. HER2 status evaluation at the DNA level

2.2.2.1. Fluorescence in situ hybridization (FISH)

The FISH technique is a cytogenetic technique that uses fluorescent probes to target specific DNA sequences in FFPE tissue samples [73]. FISH is effectuated either as a single-color assay (*HER2* probe only) to evaluate *HER2* gene copies per nucleus or as a dual-color assay using

differentially labeled HER2 and chromosome 17 centromere (chromosome enumeration probe 17, CEP17) probes simultaneously. The dual-color assay allows the determination of the HER2/CEP17 ratio [74]. The HER2/CEP17 ratio is often regarded as a better reflection of the *HER2* amplification status, as the latter may be influenced by abnormal chromosome 17 copy number (mainly polysomy) [75].

The *HER2* gene locus on chromosome 17 is recognized by the HER2 probe, which is labeled with a fluorophore (orange as example). The α satellite DNA sequence located at the centromeric region of chromosome 17 is recognized by a fluorophore-labeled chromosome 17 centromere probe (green as example). Nuclei are then counterstained with 4,6′-diamino-2-phenylindole (DAPI). Fluorescent hybridization signals can be visualized using a fluorescence microscope equipped with appropriate filters (for example Spectrum Orange for locus-specific probe HER2, Spectrum Green for centromeric probe 17, and the UV filter for the DAPI nuclear counterstain) [76].

Three FISH assay kits have been approved by the FDA for the determination of the *HER2* gene amplification in breast cancer specimens: the single-probe INFORM HER2 FISH DNA kit (Ventana Medical Systems), the dual-probe PathVysion HER-2 DNA probe kit (Abbott Molecular, Des Plaines, IL), and the dual-probe HER2 FISH PharmDx kit (DAKO).

According to the 2013 ASCO/CAP guidelines, a case is evaluated as amplified when the mean *HER2* gene copy number is ≥6 signals/nucleus or HER2/CEP17 ratio is ≥2.0, else as equivocal if mean *HER2* gene copy number is ≥4 and <6 signals/nucleus, and else as non-amplified when the mean *HER2* gene copy number is <4 signals/nucleus. In order to adequately evaluate HER2 status, a minimum of 20 tumor cell nuclei are counted in at least two invasive tumor areas. For equivocal FISH specimens, results are confirmed by counting 20 additional cells [61]. Moreover, the equivocal category requires reflex testing with the alternative assay (IHC) on the same specimen for final determination. Reflex testing can also be performed using IHC or ISH methods on an alternative specimen. If specimen is evaluated as equivocal, even after reflex testing, the oncologist may consider targeted treatment.

Although still matter of debate, several researchers consider FISH as being more accurate and reliable than IHC in the assessment of HER2 status in breast cancer specimens [77–80]. In addition, given that DNA is more stable than protein, preanalytical factors have less impact on assay results compared with IHC [81]. Although the FISH technique yields results that are considered more objective and quantitative than immunohistochemical scoring [73, 82], this method is nine times more time-consuming [83] and three times more expensive compared with IHC [84]. In addition, costly equipment is required for signal detection [67]. The FISH assay can be interpreted only by well-trained personnel, as distinguishing invasive breast cancer from breast carcinoma *in situ* under fluorescence is arduous [85].

Moreover, fluorescence signal counting is time consuming. To overcome this limitation, image analysis software for the automated assessment of fluorescence signals has been developed. Several investigators have reported an excellent concordance between HER2/CEP17 ratios calculated through manual counting and those obtained with automated image analysis system [86–88]. Some image analysis systems has been approved by the FDA for the automated determination of *HER2* gene amplification: the Metafer (MetaSystems, Altlussheim, Germany) and the Ariol HER2/neu FISH (Applied Imaging, San Jose, CA). Furthermore, this software allows the storing of captured images [86].

2.2.2.2. Bright-field in situ hybridization (ISH) methods

Given that FISH technology have some limitations, alternative ISH methods have been developed for the assessment of *HER2* gene amplification in breast cancer specimens. Similar to FISH, these methods allow the quantification of *HER2* gene copy number within tumor cell nuclei in FFPE tissues using a DNA probe that specifically recognizes specific DNA sequences. However, whereas the FISH assay is performed with DNA probes that are coupled to a fluorescent detection system, these alternative ISH methods are performed with probes that are coupled to chromogenic (chromogenic ISH [CISH]), or silver detection system (silver-enhanced ISH [ISH]), or a combination of CISH and SISH (bright-field double ISH [BDISH]) [89]. Similar to FISH, ISH methods are performed either as single-color assay or as a dual-color assay.

Since visualization is achieved using other reactions than fluorescence-labeled probe, signals can be evaluated using a standard bright-field microscope, allowing the simultaneous analysis of *HER2* gene amplification and morphologic features of tissues. Moreover, contrary to fluorescent signals that fade over time, bright-field ISH signals are permanent [90]. Here after, we will briefly describe the bright-field ISH methods that are used in clinics.

2.2.2.3. Chromogenic in situ hybridization (CISH)

CISH allows the visualization of target genes in breast cancer tissue sections through peroxidase enzyme-labeled probes [90]. The single-color CISH assay (SPOT-Light HER2 CISH kit; Life Technologies, Carlsbad, CA), and the dual-color CISH assay (HER2 CISH PharmDx kit; Dako) received FDA approval in 2008 and 2011, respectively [61].

With the single-color CISH assay, only the absolute *HER2* gene copy number is evaluated. The hybridized HER2 probe is visualized by DAB as chromogen. *HER2* gene copies are recognizable as brown chromogenic reaction product signals within nuclei. Slides are then counterstained with hematoxylin [82, 91, 92]. HER2 signals are recognizable either as large brownish signal clusters or as numerous individual brownish small signals [92]. Cases with low-level amplification show six to 10 signals per nucleus in more than 50% of breast cancer cells, whereas high-level amplification cases are characterized by a mean *HER2* gene copy number of more than 10 or by large gene copy clusters in more than 50% of breast cancer cell nuclei [92, 93].

The dual-color CISH assay allows the simultaneous visualization of the HER2 and CEP17 probes on the same slide [94]. HER2 probes are visualized using a chromogen (green as example), whereas CEP17 probes are visualized using another chromogen (red as example). Slides are then counterstained with hematoxylin. Results obtained by dual-color CISH are reported as dual-color FISH [61].

The CISH assay is twice cheaper [72] and 1.2 times faster [82] comparatively to FISH. Furthermore, since the CISH assay allows an easier identification of the invasive component compared with FISH, evaluation of CISH signals is less time-consuming than FISH [82, 94]. In addition, tumor heterogeneity is promptly recognizable, even at low magnification [95]. Moreover, the dual-color assay can be performed on an automated slide stainer, improving the reproducibility of the assay [96]. However, the assessment of *HER2* gene copy number can be arduous in tumor regions showing high-level amplification, since overlapping dots lead to formation

of signal clusters that are difficult to evaluate [94]. In addition, technical problems, including under- or overfixation, over- or underdigestion of tissue samples can lead to inaccurate results or loss of signals [91, 93].

2.2.2.4. Silver-enhanced in situ hybridization (SISH)

SISH is an automated enzyme metallography assay, in which an enzyme reaction is used to selectively deposit metallic silver from solution at the reaction site to produce a black staining [97]. All steps of the assay are performed on the Ventana BenchMark XT automated slide stainer [98, 99]. HER2 and chromosome 17 analysis is performed on sequential slides [98, 99]. As previously mentioned, HER2 and CEP17 probes are visualized through the process of enzyme metallography. During the process, silver precipitation is deposited in the nucleus, and HER2 or CEP17 signals are visualized as black dots within cell nuclei [99]. Similar to the FISH assay, *HER2* gene amplification status assessed by SISH is reported as a HER2/CEP17 ratio, according to the ASCO/CAP guidelines [61].

Given that the SISH assay is fully automated, this technique is six times faster to perform than the FISH assay [99]. In addition, black SISH signals are easier to evaluate compared with other bright-field ISH techniques [100, 101]. However, to correct for chromosome 17 aneusomy, the hybridization of a further section is required for separate assessment of CEP17 copy number [100].

2.2.2.5. Bright-field double ISH (BDISH)

Bright-field double ISH (BDISH) or dual-color *in situ* hybridization (dual ISH) is a fully automated bright-field ISH assay for the simultaneous determination of HER2 and CEP17 signals on the same FFPE breast cancer tissue sections [100]. This assay combines the visualization of *HER2* gene copies through the deposition of metallic silver particles, similar to the monocolor SISH procedure, with the detection of CEP17 copies with a red chromogen, similar to the CISH assay [102]. HER2 signals are visualized as discrete black spots and the CEP17 signals as red spots in the nuclei. Slides are then counterstained with hematoxylin [100]. *HER2* gene amplification status assessed by BDISH is reported as a HER2/CEP17 ratio, according to the ASCO/CAP guidelines.

This technique is very pertinent especially for cases displaying chromosome 17 aneusomy or intratumoral heterogeneity, as it allows the simultaneous visualization of both HER2 and CEP17 probes on the same slide [100]. Furthermore, as the HER2 signals and CEP17 signals differ in color and size (HER2 black spots are smaller than CEP17 red spots), both signals can be distinguished from each other, even though they colocalize within cell nuclei [100]. Moreover, since this assay is completely automated, results are available within 6 h, in addition of being more reproducible, as risk of human errors are diminished [101]. The BDISH assay presents the same disadvantages as CISH and SISH.

2.2.2.6. Instant-quality FISH (IQFISH) and automated HER2 FISH

Recently, new FISH assays have been developed for the evaluation of *HER2* gene amplification in breast cancer specimens, including instant-quality FISH (IQFISH), which received

FDA approval, and automated *HER2* FISH. In analogy to conventional FISH, these new assays allow the quantitative determination of *HER2* gene amplification. The IQFISH assay is performed in the same way as manual FISH, with the exception of the hybridization buffer (IQFISH buffer), which considerably reduces the time required for the hybridization step (16 times faster) and therefore the total assay time [103, 104]. Moreover, while hybridization buffer provided in conventional FISH assay contain the toxic formamide, the IQFISH buffer is nontoxic [103]. Compared to conventional FISH, automated FISH is less expensive, since the full automation of the assay requires less human intervention [105]. Furthermore, automated FISH enables faster processing of samples and recording [105].

2.2.3. HER2 status evaluation at the RNA level

2.2.3.1. Polymerase chain reaction (PCR)-based assays

Polymerase chain reaction (PCR) is a technique used for the detection of DNA samples through the exponential amplification of target DNA sequences.

Reverse transcription PCR (RT-PCR) assay allows the quantification of mRNA and can be used for the evaluation of HER2 expression in breast cancer specimens in both FFPE and frozen tissues [106, 107]. Extracted mRNA is at first reverse transcribed into complementary DNA (cDNA). cDNA is then measured by quantitative PCR (qPCR). The relative quantitation of *HER2* gene expression is evaluated comparing the target gene expression with that of housekeeping genes. The relative *HER2* gene expression measured in samples is then normalized to a calibrator obtained by mixing RNA from several normal breast tissue specimens. Of note, the Oncotype Dx (Genomic Health, Redwood City, CA) assay is a test based on RT-PCR technology and is used to analyze the expression of 21 genes involved in breast cancer biology, such as *HER2*, ER, and PR. This assay is used to predict the likelihood of breast cancer recurrence in patients with early-stage, node-negative, ER-positive breast cancer [106].

RT-PCR has a large dynamic range, in addition of being a quantitative method. PCR results, however, are often associated with false-negative results due to dilution of amplified tumor cells with surrounding nonamplified stromal cells [108, 109]. In addition, the evaluation of HER2 status at the mRNA level by RT-PCR using FFPE tissues can be problematic, as mRNA integrity can be damaged by several factors, including tissue fixation and storage time [110].

3. Conclusion(s)

HER2 is a prognostic marker in breast cancer. HER2 overexpression and *HER2* gene amplification, which occur in 15–20% of breast cancer patients, cause aberrant constitutive activation of the signaling pathway. This leads to uncontrolled and unregulated cell growth and correlates with poor outcome of HER2-positive breast cancer patients.

In addition, HER2-positive status is considered a predictive marker of response to HER2-targeted drugs, including trastuzumab and lapatinib [111]. Considering the clinical and economic implications of targeted anti-HER2 treatments, reliable HER2 test results are essential.

False negative results would deny the patients access to the potential benefits of trastuzumab, whereas false positive results would expose patients to the potential cardiotoxic side effects of this expensive agent without experiencing any therapeutic advantages [89].

Although several techniques have obtained FDA approval for the HER2 assessment in breast cancer specimens, the ASCO/CAP guidelines recommend performing IHC or ISH methods to determine HER2 status in breast cancer. The optimal method for evaluating HER2 status in breast cancer specimens, however, is still matter of debate, since each method is characterized by its own advantages and disadvantages. Therefore, emphasis must be put on standardization of procedures and quality control assessment of already existing methods. Also, development of new accurate assays should be promoted. Moreover, large clinical trials are needed to identify the technique that most reliably predicts a positive response to HER2 inhibitors.

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Conflict of interest

The authors have no conflicts of interests to declare.

Notes/thanks/other declarations

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Acronyms and abbreviations

HER2	Human epidermal growth factor receptor 2
GRB7	Growth factor receptor bound protein 7
ESR1	Estrogen Receptor 1
PGR	Progesterone Receptor
FDA	Food and Drug Administration
EGFR	Epidermal growth factor receptor

IHC	Immunohistochemistry
FISH	Fluorescence *in situ* hybridization
ERBB2	erb-b2 receptor tyrosine kinase 2
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
ATP	Adenosine triphosphate
ECD	extracellular domain
EGF	Epidermal growth factor
EPG	Epigen
TGFα	Transforming growth factor α
PI3K	Phosphatidylinositol 3′-kinase
ERK	Extracellular signal-regulated kinase
MAPK	Mitogen-activated protein kinase
FGFR	Fibroblast growth factor receptor
IR	Insulin Receptor
VEGFR	Vascular Endothelial Growth Factor Receptor
Cdk	Cyclin-dependent kinase
FFPE	Formalin-fixed, paraffin-embedded
DAB	3,3′-diaminobenzidine tetrahydrochloride
ASCO	American Society of Clinical Oncology
CAP	College of American Pathologists
ELISA	Enzyme-linked immunosorbent assay
CEP17	Chromosome enumeration probe 17
DAPI	4,6′-diamino-2-phenylindole
ISH	*in situ* hybridization
CISH	Chromogenic *in situ* hybridization
SISH	Silver-enhanced *in situ* hybridization
BDISH	Bright-field double ISH
PCR	polymerase chain reaction

RT-PCR	Reverse transcription PCR
cDNA	Complementary DNA
qPCR	Quantitative PCR

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Chapter 3

Is Melanoma a Hormone-Dependent Cancer or a Hormone-Responsive Cancer?

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Additional information is available at the end of the chapter

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Abstract

Melanoma, a potentially fatal form of skin cancer is on the rise. This review not only underlines the close connection between skin and endocrine system, but also lists evidences from multiple sources epidemiological, clinical, previous in vivo and in vitro studies regarding the involvement of sex steroids in melanoma. Incidentally, clinical studies underscored the involvement of sex steroids in the protective function in melanoma in menstruating females. But, none of these studies identified the sex steroids involved in the protective function in melanoma in menstruating females. The sex steroid involved in this innate protection in melanoma in menstruating females has not been investigated by scientists, though advances have been made in immunotherapy with accompanying side effects. In this context, our in vitro studies on mouse and human melanoma cell lines, along with literature survey, pointed to progesterone as the possible female sex steroid involved in the protective function in melanoma. Based on our findings and previous studies, it is concluded in this review that melanoma is not a hormone-dependent cancer. But, it may be a hormone-sensitive or responsive cancer, as hormones (sex steroids) inhibited melanoma cell proliferation in vitro. This new understanding will help in developing new therapy or target for melanoma treatment.

Keywords: melanoma, epidemiological studies, clinical studies, in vivo and in vitro studies, protective function, progesterone, hormone-responsive cancer

1. Introduction

The question that has been raised over the years, whether melanoma is a hormone-dependent cancer or not still lacks a clear-cut answer [1–4]. However, in this review, an attempt has been made to collect evidences from multiple sources to point out the nature of melanoma. Melanoma, the fatal form of skin cancer accounts for less than 2% of skin cancer, but it is

responsible for 75% of deaths due to skin cancer [5]. According to the American Cancer Society reports known as Cancer Statistics, melanoma is on the rise. In 2018, in the United States, 91,270 new cases will be diagnosed with an estimated 9320 deaths [6]. Melanoma occurs mostly on the skin [7]; however, some rare forms of melanoma can occur in other areas such as cornea, uvea, and gastrointestinal tract [7]. Epidemiological data indicated an increased mortality rates in males than in females [8], suggesting a sex difference. Clinical studies showed that menstruating females were better protected (delayed metastasis and increased survival) in melanoma than postmenopausal women and men of any age [9], clearly indicating the role of sex steroid hormones in the protection function. It is important to point out that skin itself functions as an endocrine organ [10], even though it is not acknowledged as one. Skin possesses many of the enzymes necessary for synthesis of steroid hormones [11]. In fact, most of the peripheral conversions of dehydroenpiandrosterone (DHEA) and androstenedione (AD) to testosterone and estradiol take place in the skin [12]. This area of endocrinology is known as intracrinology [12]. In addition, skin is also a target organ for various hormones. Sex steroids such as androgens, estrogens, and progestins are essential for a healthy skin [13]. Melanocyte, which is transformed to melanoma cell is also under the influence of melanocyte-stimulating hormone (MSH) from pituitary [14]. Hence, it is natural to ask the question whether melanoma is a hormone-dependent cancer like breast, prostate, and endometrial cancers. Generally, melanoma is not labeled as a hormone-dependent cancer because of the belief that UV rays from the Sun is the major cause for melanoma [15]. UV rays cause DNA damages and other inflammatory changes in the skin, which result in skin cancer. About 90% of melanoma is caused by environmental factors such as UV rays, radiations, and only 10% is inherited in the family. So, melanoma is never considered as a hormone-dependent cancer. However, existing evidences point to a hormone relatedness or a hormone-responsive nature of melanoma cancer:

1. Evidences of relationship between skin and endocrine system: there is a close connection between skin and endocrine system, as shown by the following examples.
    a. All the components of a functional hypothalamo-pituitary-adrenal axis analog are present in the skin.
    b. Presence of enzymes involved in steroid hormone synthesis in skin cells: the level of local steroid production depends on the expression of androgen- and estrogen-synthesizing enzymes present in specific cell types. Five major enzymes are involved in the activation and deactivation of androgens in the skin [13].
    c. Actions of sex steroid hormones on skin: skin is a target organ for sex steroid hormones. Androgens are essential for differentiation and growth of Sebocyte and hair growth. Estrogen is responsible for skin pigmentation and skin cancer. Progesterone functions, though not clear is essential for treating acne [13].
    d. Endocrine disorders manifested on the skin:
        i. Association of insulin resistance and metabolic syndrome with acne: post-adolescent male patients with acne more commonly have insulin resistance [16]. This resistance may be a stage of prediabetes and the patients may develop hyperinsulinemia or type 2 diabetes in the future.

ii. Association of cutaneous findings and systemic abnormalities in women suspected of having polycystic ovary syndrome (PCOS): Hirsutism and acne are the most reliable cutaneous markers of PCOS and require a comprehensive skin examination to diagnose [17].

iii. Psoriasis severity may influence type 2 diabetes risk: people living with psoriasis are not only at higher risk of type 2 diabetes, but their risk also rises in line with the skin disease's severity [18].

It is evident from the above mentioned examples that skin is not only an endocrine organ which produces various hormones, but also has a close relationship with systemic endocrine system.

2. Evidences from epidemiological data

According to the epidemiological SEER data [8] known as Surveillance, Epidemiology and End Results program, a database maintained by NCI, there has been an increase in the incidence of melanoma. The incidence of melanoma ('03-'07) for men and women were 26.7 per 100,000 and 16.7 per 100,000 respectively. There has been an increase in death rate also. Even in the death rate, there was a difference between males and females. The mortality rate for males was 4.0 per 100,000, whereas for females it was 1.7 per 100,000. Males have increased mortality rate than females. Death rate was cut more than half in females. Similarly, malignant melanoma database (1971–2012) maintained by UK Cancer Research Council [19] also showed that the morality rate was higher in males than in females over the years. The data [20] was almost similar from Australian continent, where the incidence of melanoma is the highest in the world. From 1982 to 2016, the number of melanoma diagnosed in Australia increased from 3526 to an estimated 13,280. The age-standardized incidence rate increased for both males and females, from 28 to 60 cases per 100,000 males, and from 26 to 39 cases per 100,000 females. Data showed males were more prone to melanoma than females [20]. Thus epidemiological data from three continents clearly showed that males were more affected by melanoma than females. These gender differences in melanoma demand an investigation of the effect of sex hormones on this malignancy.

3. Evidences from clinical studies

Clinical studies supported the epidemiological findings. Clinical studies showed that menstruating females were better protected (delayed metastasis and increased survival) in melanoma than post-menopausal women and men of any ages [9]. This very difference between menstruating females and postmenopausal women clearly indicated the involvement of steroid hormones in protecting menstruating females in melanoma. However, these data base were not correlated with the steroid status of females. Studies published between 1977 and 1966 showed women had better survival in all but 4 out of 22 epidemiologic studies [21]. Two female hormones could be involved in rendering protection, namely estrogen and progesterone. First, estrogen as the hormone protecting menstruating females in melanoma: estrogen receptor antagonist tamoxifen was evaluated as a single agent in 12 studies covering 213

patients with metastatic melanoma cancer [22]; the response rate was only 7%. Moreover, estrogen receptors were found in some cancers only by biochemical and histochemical tests but not by the immunohistochemical tests using monoclonal antibodies [23]. Second, progesterone as the possible female sex hormone involved in the protection: there were only limited in vitro studies [24, 25] and they were also not tied to the protective function in melanoma. According to the data published on pregnancy and melanoma, several studies reported statistically no significant differences in survival rates between controls (non-pregnant women with malignant melanoma) and women diagnosed with melanoma stage I or II during pregnancy [26–28]. Studies also found no association between melanoma and oral contraceptives [29, 30]. Data on the relationship between melanoma and hormone replacement therapy were meager and it seemed that exogenous hormones did not influence the risk for malignant melanoma [31, 32]. So, clinical studies underlined the involvement of female sex steroid hormones in protecting menstruating females in melanoma. But, these clinical studies did not identify the exact female hormone involved in the protection.

4. Evidences from animal studies

Animal studies also showed the involvement of sex steroid hormones in the regulation of melanoma growth and there were also differences in the regulation of melanoma growth between male and female mice.

a. Female survival benefit with metastatic melanoma was observed, when melanoma cells produced liver metastases preferentially in male compared to female mice [33].

b. In another study, estrogen receptor-positive human melanomas cells grew more slowly in females than in males mice [34].

c. Similarly, dihydrotestosterone was shown to stimulate proliferation. But, in a follow-up study, male mice transplanted with melanoma showed increased survival after treatment with anti-androgen receptor hydroxyflutamide [35].

d. Male mice were significantly more susceptible to carcinogen-induced skin cancer than female mice [36].

e. Similarly male mice were more susceptible to UV-B induced skin carcinogenesis than female mice [37]

f. Research work presented in one study showed that metapristone (a metabolite of mifepristone (RU-486)) had a remarkable effect of preventing cancer metastasis of B16-F10 cells in vivo compared with mifepristone [38].

5. Evidences from previous cell-culture studies

Apart from epidemiological, clinical, and in vivo animal studies, various in vitro studies using a variety of melanoma cell lines showed the inhibitory effect of steroid hormones on melanoma cell growth, suggesting melanoma could be a hormone-sensitive or responsive cancer.

a. In one study, 2-methoxyestradiol (2-ME), an estrogenic metabolite inhibited the growth of all melanoma cells tested, without inhibiting the growth of non-tumorigenic cells [39].

b. Data from another study suggested that 17-β-estradiol, progesterone, and dihydrotestosterone suppressed the growth of melanoma cells by inhibiting interleukin-8 production in a receptor-dependent manner [25].

c. However, Feucht et al. investigated three human melanoma cell lines and found no effect either by estradiol or tamoxifen on melanoma cell growth in vitro [34].

d. Another in vitro study indicated a direct inhibitory effect of testosterone on growth of an amelanotic strain which in vivo grew faster in female hamsters [40].

e. The findings in another study indicated that glucocorticoids exerted some influence on the growth of human melanoma cells and this effect was mediated through glucocorticoid receptor [41].

f. Only study which showed a stimulatory effect was with melanocyte, where α-MSH stimulated melanocyte proliferation in a dose-dependent manner, but its stimulatory effect required bFGF and/or the activation of protein kinase C [42].

g. Another in vitro study showed that melatonin at physiological concentrations (1 nM to 10 pM) inhibited metastatic mouse melanoma (B16BL6) cell growth [43].

6. Evidences from our studies on mouse melanoma (B16F10) cell line

a. Progesterone effect on mouse melanoma (B16F10) cell growth: based on previous research work and literature survey, initially four sex steroids were checked for their effect on mouse melanoma (B16F10) cell growth [44]. Of the four steroids checked [dehydroepiandrosterone (DHEA), androstenedione (AD), testosterone (T) and progesterone (P)], progesterone showed significant inhibition (87%) of mouse melanoma cell growth. Though other steroid hormones also showed inhibition of cell growth, but it was not as significant as that of progesterone inhibition (**Figure 1**).

b. Progesterone dose-response study with mouse melanoma cells: as the initial experiment was carried out at high concentrations (100, 150, 200 μM) of hormones, a follow-up dose-response study was carried out with progesterone alone. Dose-curve study with progesterone showed a dose-dependent decrease in mouse melanoma cell growth (**Figure 2**).

c. Further studies with mouse melanoma cell line: further studies (data not shown) showed that the effect of progesterone on mouse melanoma cells was not a toxic, not a spurious or not a non-specific effect [44]. The only other steroid which showed a significant inhibition of mouse melanoma cell growth was progesterone-receptor antagonist RU-486, a synthetic steroid (**Figure 3**).

d. Mechanism of progesterone action on mouse melanoma cell line: since RU-486 also showed a dose-dependent inhibition of mouse melanoma cell growth, it was decided to find out whether the actions of progesterone and RU-486 were mediated through progesterone

receptor. A co-incubation study was carried out with fixed concentration of progesterone (50 μM) and varying concentrations of RU-486 (10, 50, 100 μM). Co-incubation study showed an additive effect (data not shown) on mouse melanoma cell growth suggesting that the action was not mediated through progesterone receptor [44].

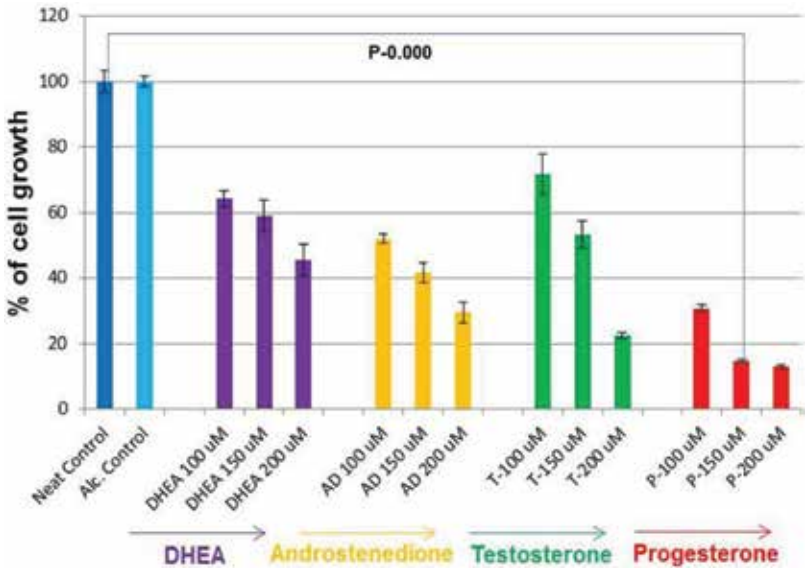

Figure 1. Various steroid hormones effect on mouse melanoma (B16F10) cell growth: three androgens (DHEA, AD, T) and one female sex steroid hormone (P) were checked for their effect on mouse melanoma cell growth at 100, 150, and 200 μM concentrations. Cells were incubated with the hormones separately for 48 h. After 48 h, cell growth was assessed by MTT assay. All the steroids checked showed dose-dependent decrease in cell growth. But, progesterone showed a significant inhibition of cell growth (87%) at 200 μM concentration.

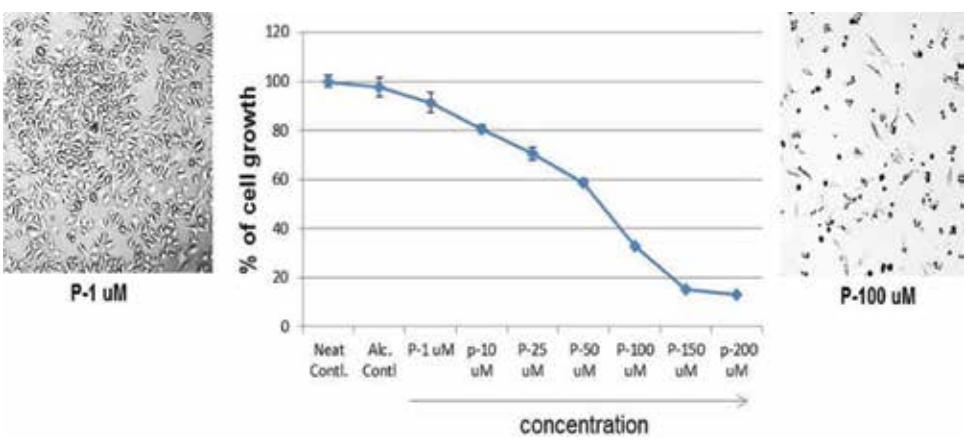

Figure 2. Dose-response study with mouse melanoma cell line: since the initial study was carried out at high concentrations, a dose-response study was carried out with progesterone starting from 1 to 200 μM. Progesterone showed a dose-dependent decrease in mouse melanoma cell growth with significant inhibition at 200 μM concentration. Cell growth was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

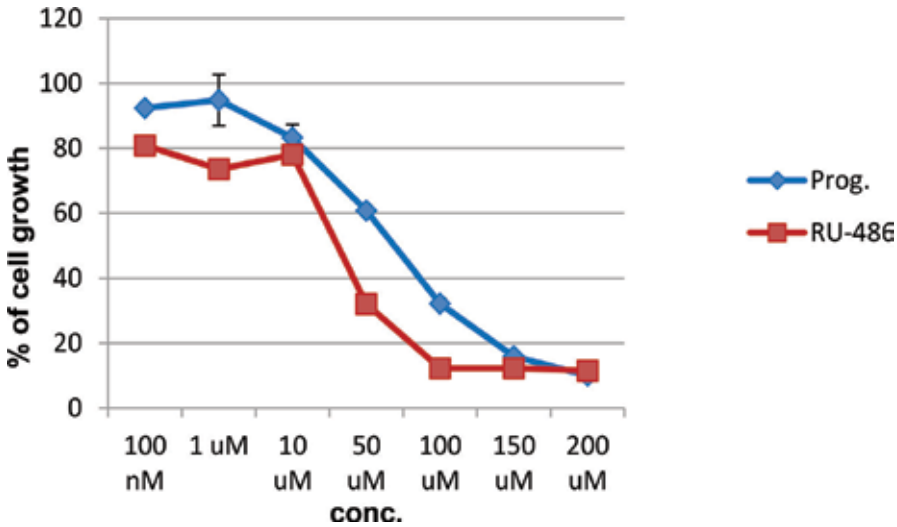

Figure 3. Comparison of dose-response curves of progesterone and RU-486: both progesterone and RU-486 were treated separately for 48 h. After 48 h, cell growth was assessed by MTT assay. Dose-response curve of RU-486 was compared with that of progesterone. The dose–response curves were very similar to each other.

7. Evidences from our studies on human melanoma (BLM) cell line

a. Dose-response study with progesterone and RU-486 on human melanoma cell line: the sex steroids (progesterone and RU-486), which showed inhibition on mouse cell line were checked on human melanoma (BLM) cell line for their effect [45, 46]. Progesterone and RU-486 also showed a dose-dependent inhibition of human melanoma cell growth (**Figure 4**).

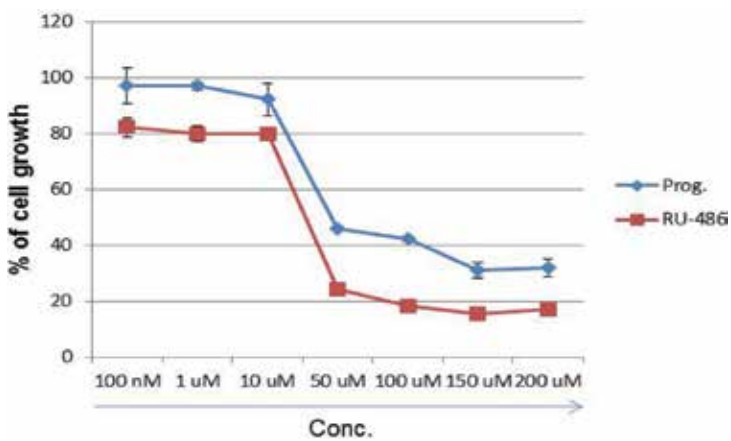

Figure 4. Dose-response study of progesterone and RU-486 on human melanoma cell line: progesterone and RU-486 were incubated separately with human melanoma cells for 48 h. After 48 h of incubation, cell growth was measured by MTT assay. Dose-response study of both progesterone and RU-486 showed a dose-dependent decrease of human melanoma cell growth.

b. Mechanism of action of progesterone on human melanoma cells: since progesterone and RU-486 separately showed a dose-dependent inhibition of human melanoma cell growth, it was decided to find out whether these actions were mediated through progesterone receptor on human melanoma cell line. Therefore, co-incubation study, just like the one on mouse melanoma cell line was carried out. Co-incubation study showed that the effect was not mediated through progesterone receptor (**Figure 5**). In fact, co-incubation of the two steroids (progesterone and RU-486) showed an additive effect on cell growth inhibition, suggesting the actions were mediated through two different mechanisms.

c. Mechanism of inhibition of human melanoma cell growth by progesterone: since the co-incubation study suggested that the mechanism of action of progesterone and RU-486 could be different, it was decided to find out the mechanism of inhibition of human melanoma cell growth by progesterone. After having ruled out necrosis and apoptosis as the mechanism of inhibition of cell growth, it was found out that autophagy was the cause for cell growth inhibition by co-incubating progesterone and 3-methyl adenine (3-MA) on melanoma cells. 3-methyl adenine (3-MA) had been used in various studies to check or

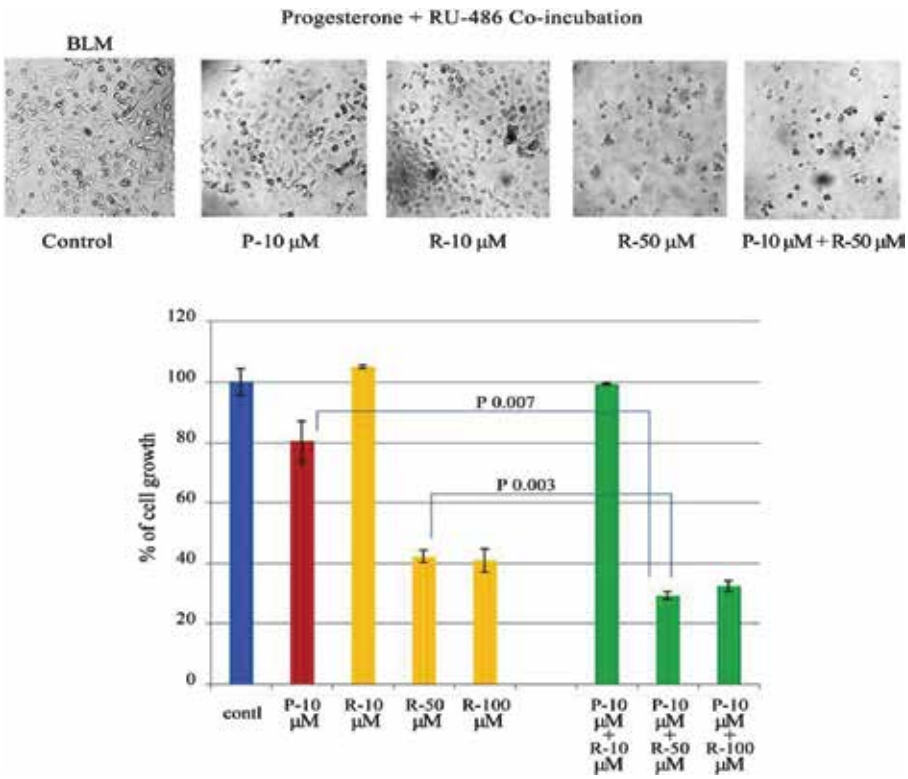

Figure 5. Co-incubation of progesterone and RU-486: a fixed concentration of progesterone (10 μM) was co-incubated with varying concentrations of RU-486 (10, 50, 100 μM). Co-incubated cells showed an additive effect on cell growth inhibition, suggesting the action was mediated through different mechanisms and not through progesterone receptor.

inhibit autophagy [47–49]. Therefore, the mechanism of inhibition of human melanoma cell growth by progesterone was due to autophagy (**Figure 6**).

d. Suppression of adhesion and migration functions of human melanoma cells by progesterone: metastasis of cancer involves adhesion, migration, and invasion functions. Progesterone ability to suppress metastasis was checked by in vitro adhesion and migration assays after treatment with progesterone for 48 h [50]. Progesterone at 100-μM concentration decreased adhesion function to 71% compared to untreated control cells at 100%. Similarly progesterone at 50 μM significantly decreased migration to 20% compared to untreated control cells at 100%. Adhesion and migration assays suggested that progesterone could be playing a role in delayed metastasis, as reported in clinical studies [9] (**Figure 7**).

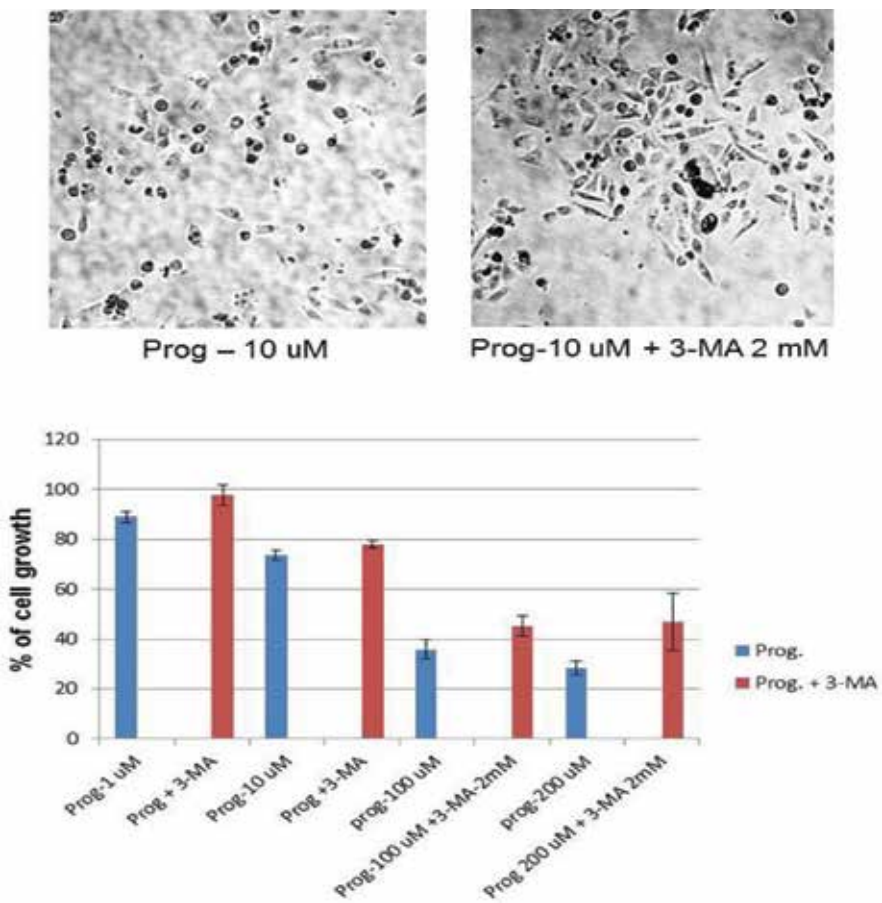

Figure 6. Mechanism of human melanoma (BLM) cell growth inhibition by progesterone: after having ruled out necrosis and apoptosis as mechanism of inhibition, it was decided to find out whether autophagy was the mechanism of inhibition of cell growth. So, cells were co-incubated with progesterone and 3-MA (2 mM) for 48 h. After 48 h, cell growth was monitored by MTT assay. 3-methyl adenine (3-MA) partially rescued melanoma cell growth, showing a slight increase in co-incubated cell growth compared to progesterone alone treated cell growth. 3-methyl adenine (3-MA) had been shown to disrupt the formation of autophagsome/lysosomal degradation in various studies [47–49].

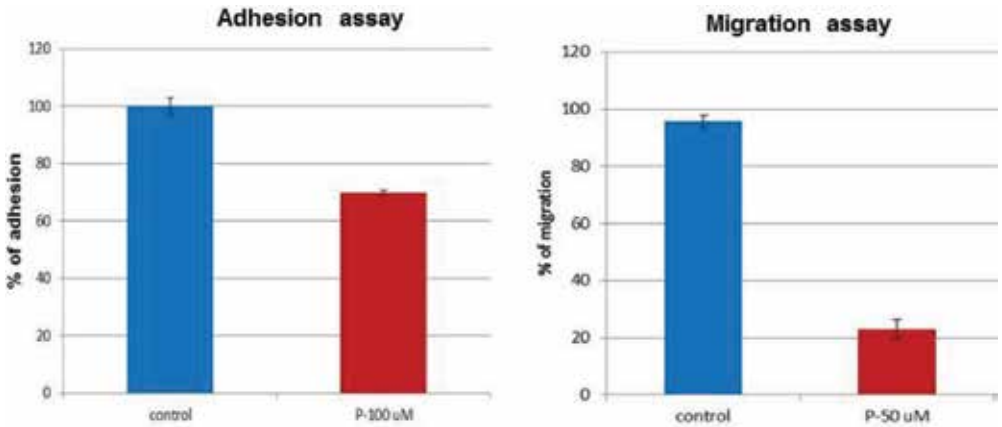

Figure 7. Suppression of adhesion and migration functions by progesterone: human melanoma cells were treated with progesterone for 48 h in petri dishes. After 48 h, cells were harvested from control and progesterone treated cells. Adhesion assay was carried out in a 96 well plate with 30,000 cells/wells. Cells were incubated for 60 min and washed. Cells attached to the plate were fixed with 2% paraformaldehyde and stained with 0.2% crystal violet dye. Purple color dye was eluted with isopropanol and assayed at 570 nm in a plate reader. For migration assay, treated cells were placed in a 24 well plate and allowed to become confluent. Cells were scratched in the middle of the plate with a tip, which was considered as 0 time point and cells were allowed to incubate for 24 h. After 24 h, percentage of cells migrated to the cleared space was calculated with a software.

8. Summary

There is a close connection between skin and endocrine system, as shown by the neuroendocrine properties of skin. Skin not only functions as an endocrine organ but also as a target organ for various hormones. Epidemiological studies highlighted the differences in mortality rate between males and females and hinted the involvement of hormones in melanoma. Clinical studies pinpointed the role of sex steroids, mainly female sex steroids, in melanoma. Animal studies also highlighted the involvement of sex steroids in melanoma. In vitro studies with steroid hormones showed inhibition of melanoma cell growth. Our studies showed the in vitro effect of progesterone on mouse and human melanoma cell growth. In our studies, progesterone showed significant inhibition of mouse and human melanoma cell growth. The mechanism of inhibition was due to autophagy and the effect was not mediated through progesterone receptor. In vitro study also showed suppression of adhesion and migration functions after progesterone treatment, suggesting progesterone could be involved in delayed metastasis of cancer. This in vitro finding supported the clinical studies which showed menstruating females (whose progesterone level vary between 1000 and 1500 ng/dL) were better protected in melanoma than post-menopausal women (whose progesterone level vary between 20 and 100 ng/dL) and men of any age. A similar study with different human melanoma (A375, A875) cell lines by Fang et al. [51] also showed that progesterone and RU-486 inhibited melanoma cell growth and this effect was also not mediated through progesterone receptor. Similar result was observed in another study using progesterone and the same melanoma cell lines by Moroni et al. [31]. Kanda and Watanbe [25] had already shown the inhibition of human melanoma cells by progesterone. Thus the inhibition of melanoma cell growth

by progesterone was observed by 3 other different groups. Thus, the preceding studies, our own studies, and previous studies by others lend support to the idea that melanoma is amenable to hormone action and that melanoma is sensitive or responsive to steroid hormones.

9. Conclusion

Evidences from multiple sources (epidemiological, clinical, in vivo, and in vitro) suggested the involvement of hormones in melanoma and that melanoma was amenable to hormone action. But, unlike breast, ovary, and prostate cancers, addition of hormones did not stimulate proliferation of melanoma cells, suggesting melanoma was not a hormone-dependent cancer. However, addition of hormones suppressed melanoma cell proliferation, suggesting melanoma might be a hormone-sensitive or responsive cancer. Therefore, acquisition of melanoma may not be hormone dependent, but survival (suppression of cancer cell proliferation) in melanoma may be hormone dependent. Hence, based on epidemiological findings, clinical studies, literature reports of previous in vivo and in vitro experiments, and our own experiments, melanoma may be considered as a hormone-sensitive or responsive cancer. This understanding will help in generating new therapy or therapeutic target for melanoma treatment.

Declaration of interest

Author has nothing to declare.

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Section 3

Prognosis and Diagnosis Factors Studies

CBX4 Expression and AFB1-Related Liver Cancer Prognosis

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Additional information is available at the end of the chapter

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Abstract

Background: Previous studies have shown that chromobox 4 (CBX4) expression may involve in the progression of liver cancer, however, it is unclear whether it affects the prognosis of hepatocellular carcinoma (HCC) related to aflatoxin B1 (AFB1).

Methods: A retrospective study was conducted in the high AFB1 exposure areas and a total of 428 patients with HCC were included in the final survival analyses. AFB1 exposure levels and CBX4 expression in the tumor tissues were tested using enzyme-linked immunosorbent assay and immunohistochemistry, respectively. The effects of AFB1 and CBX4 on HCC outcome were elucidated by Kaplan–Meier survival method and Cox regression model.

Results: We found that the levels of AFB1 exposure and CBX4 expression in tumor tissues were significantly associated with some clinicopathological features such as microvessel density and tumor stage. Furthermore, both AFB1 and CBX4 significantly modified overall survival and tumor reoccurrence-free survival status of HCC. Additionally, some evidence of CBX4-AFB1 interaction affecting HCC prognosis was observed, with an interactive value of 1.98 for overall survival and 1.94 for tumor reoccurrence-free survival, respectively.

Conclusion: These results suggest that CBX4 expression might be a useful marker for AFB1-related HCC prognosis.

Keywords: CBX4, AFB1, HCC, prognosis

1. Introduction

Aflatoxin B1 (AFB1) is a type of secondary metabolite of *Aspergillus parasiticus* and *Aspergillus flavus*, and frequently contaminates a series of staple foods, such as ground nuts, and maize [1–3]. Once this type foods contaminated by AFB1 entered into human bodies, it is metabolized into its epoxides consisting of AFB1–8,9-exo-epoxide (AFBEX) and AFB1–8,9-endo-epoxide (AFBEN) by cytochrome P450 (CYP) metabolic system [3]. These products of AFB1, especially AFBEX, are characterized by high reaction, genic toxicity, and carcinogenicity [3]. Evidence from molecular epidemiology and animal models has shown that AFB1 is an important carcinogen inducing hepatocellular carcinoma (HCC) [4–10]. Mechanically, the carcinogenesis of AFB1-related HCC mainly involves in the formation of DNA damage (including AFB1-DNA adducts, DNA single-strand breaks, DNA double-strands breaks, and gene mutations), the inactivation of such tumor suppressor gene as TP53, and the activation of cancer genes such as Ras [3, 11–15]. Although some advance in the pathogenesis of AFB1-related HCC has obtained in the past decades [16–18], it is still far for us to elucidate more detailed mechanisms.

The chromobox 4 (Cbx4) (GenBank accession NO. 8535) consists of six exons and spans about 6.26 kb on chromosome 17q25.3. This gene encodes a 560-amino acid protein which is the important component of polycomb repressive complex 1 (PRC1) [19–22]. Functionally, CBX4 involves in PRC1-regulated transcription repression and post-translation modification [19–22]. Recently, increasing evidence has exhibited that the dysregulation of this gene may affect the carcinogenic process of some tumors such as HCC, colorectal cancer, breast cancer, and so on, and may be a significant prognostic biomarker [19, 21, 23–29]. However, it is not clear whether CBX4 modify the prognosis of AFB1-related HCC. Here, we conducted a hospital-based retrospective study to investigate whether the CBX4 expression in the cancerous tissues is associated with the outcome of HCC related to AFB1 expression in the Guangxi Region, a high AFB1 exposure area.

2. Materials and methods

2.1. Study population

Between January 2009 and December 2012, 428 consecutive patients with histopathologically confirmed hepatocarcinoma were recruited at the Divisions of Oncology and Pathology, the affiliated Hospitals of Guangxi Medical University and Youjiang Medical University for Nationalities. During the recruitment phase, only 5 cases refused to participate in the study (response rate 98.8%). All cases were from high AFB1 exposure areas, including Nanning, Bose, Tiandong, and Tianyang. After informed consent was obtained, surgically removed tumor samples were collected to analyze the amounts of AFB1-DNA adducts and CBX4 protein in the cancerous tissues. Additionally, all corresponding clinicopathological and survival following-up data were also collected in the hospitals as previously described methods [30-32]. In this study, the status of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection was evaluated using serum hepatitis B surface antigen (HBsAg) and anti-HCV, respectively; whereas the grade and stage of tumor was elucidated using the Edmondson and Steiner (ES) grading system and the Barcelona Clinic Liver Cancer (BCLC) staging system, respectively. For survival analyses, the

last follow-up day was set on December 31, 2017. The study protocol was carried out according to the approved guidelines by the Institutional Ethics Committee from the Affiliated Hospitals of Youjiang Medical University for Nationalities and Guangxi Medical University.

2.2. Microvessel density (MVD) assay

MVD in the cancerous tissues was assessed using the immunohistochemistry staining of CD31 as our previously descripted [30]. In this study, positive status of MVD was defined as microvessel counts more than 50 per ×200 magnifications.

2.3. AFB1 exposure data

AFB1 exposure levels were evaluated using the amounts of AFB1-DNA adducts in the cancerous tissues as our previously descripted [31, 32]. The amounts of AFB1-DNA adduct were tested using the competitive enzyme-linked immunosorbent assay. In this study, a value than less 1.00 μmol/mol DNA was considered as negative status for AFB1 exposure.

2.4. CBX4 expression assays

The level of CBX4 protein expression in cancerous tissues was elucidated using our previously published immunohistochemistry method [33, 34]. Briefly, the amounts of CBX4 protein were tested using anti-CBX4 antibody and calculated using immunoreactive score system (IRS). In the present study, positive CBX4 protein in cancerous tissues was define as IRS > 4.

2.5. Statistical analysis

Logistic regression model with enter method for variables (including all known clinicopathological features) was used for statistical comparison between groups. The odd ratios (ODs) and corresponding 95% confidence intervals (CIs) were calculated in this model for evaluating the association between clinicopathological features of HCCs and either AFB1 exposure or CBX4 expression. Kaplan–Meier survival method with log-rank test was used for statistical comparisons between different levels of AFB1 expression and CBX4 expression. Multivariate Cox regression model (with retread method based on likelihood ratio test) analyses were performed to calculate the risk strength of independent variates and prognostic values. In this study, all analyses were finished using the SPSS soft version 18.0 (SPSS Inc. Chicago, IL), and a *P*-value less than 0.05 was defined as statistical significance.

3. Results

3.1. The clinicopathological and survival features of HCC cases

Table 1 gave the clinicopathological characteristics of all cases, and a total of 428 patients with HCC were included in the final analyses. All cases were followed-up more than 5 years to obtain median survival time. During the follow-up period, 261 patients with

Variables	n	%
Total	428	100.0
Age, years		
Mean ± SE	47.9 ± 10.1	—
Range	30–75	—
Sex		
Man	290	68.9
Female	138	32.8
Ethnicity		
Han	229	54.4
Zhuang	199	47.3
HBV status		
HBsAg (−)	113	26.8
HBsAg (+)	315	74.8
HCV status		
anti-HCV (−)	378	89.8
anti-HCV (+)	50	11.9
Smoking status		
No	315	74.8
Yes	113	26.8
Drinking status		
No	304	72.2
Yes	124	29.5
AFP (ng/mL)		
≤ 20	154	36.6
> 20	274	65.1
Liver cirrhosis		
No	104	24.7
Yes	324	77.0
BCLC stage		
A	167	39.7
B	121	28.7
C	140	33.3
Tumor size		
≤ 3 cm	211	50.1
> 3 cm	217	51.5
MVD		
Negative	192	45.6

Variables	n	%
Positive	236	56.1
ES grade		
Low	226	53.7
High	202	48.0

Abbreviations: AFP, α-fetoprotein; BCLC, the Barcelona Clinic Liver Cancer staging system; ES, Edmondson and Steiner grading system; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; MVD, microvessel density.

Table 1. The clinic-pathological features of cases with hepatocellular carcinoma.

HCC featured cancer recurrences with 30.00 (22.20–37.80) months of median recurrence-free survival time (MRT), and 270 died with 45.00 (38.98–51.02) months of median overall survival time (MST).

3.2. The effects of AFB1 exposure on the clinicopathological features and the prognosis of HCC cases

In this study, the status of AFB1 exposure was elucidated using the amount of AFB1-DNA adducts in the cancerous tissues. Results from competitive ELISA exhibited the patients with HCC featured a 2.82 ± 1.60 μmol/mol DNA of AFB1 exposure level. To investigate the effects of AFB1 exposure on the clinicopathological features of HCC cases, we defined the amount of AFB1-DNA adducts ≤1.00 μmol/mol DNA as negative AFB1 exposure according to our previous published results [31, 32]. Our results showed that these patients with positive AFB1 status (AFB1-DNA adducts: > 1.00 μmol/mol DNA) had higher BCLC stage (adjusted OR = 2.09 and adjusted 95% CI = 1.04–4.24), bigger tumor size (adjusted OR = 69.06 and adjusted 95% CI = 33.62–141.86), and higher MVD (adjusted OR = 2.56 and adjusted 95% CI = 1.36–4.81) compared with those without positive AFB1 status (OR = 1) (**Table 2**). Additionally, we also found that the levels of AFB1 exposure were significantly associated with the age of patients with hepatocarcinoma (adjusted OR = 1.80, adjusted 95% CI = 1.22–2.66, and $P = 3.07\times10^{-3}$). However, AFB1 exposure was not correlated with other clinicopathological features of HCCs (**Table 2**).

Next, we investigated the effects of AFB1 exposure on the HCC prognosis using Kaplan–Meier survival model (**Figure 1A**). Results exhibited that HCC cases with negative AFB1 status (AFB1-DNA adducts: ≤ 1.00 μmol/mol DNA) featured longer median overall survival time (MST) [69.00 (55.41–82.59) months] and median tumor reoccurrence-free survival time (MRT) [70.00 (44.93–95.07) months] compared with those with positive AFB1 status [20.00 (13.04–26.96) months for MST and 13.00 (9.54–16.46) months for MRT, respectively].

3.3. The effects of CBX4 expression on the clinicopathological features and the prognosis of HCC cases

In the present, the levels of CBX4 protein in the cancerous tissues were amounted using immunohistochemistry technique with IRS counting system and the median IRS value was 5.58 for

	AFB1 (–)		AFB1 (+)			
Variables	**n**	**%**	**n**	**%**	**OR (95% CI)**	P_{trend}
Total	244	100.0	184	100.0	—	—
Age (years)						
≤ 48	148	60.7	86	46.7	Reference	
> 48	96	39.3	98	53.3	1.80 (1.22–2.66)	3.07×10^{-3}
Sex						
Man	160	65.6	130	70.7	Reference	
Female	84	34.4	54	29.3	1.13 (0.59–2.13)	0.72
Ethnicity						
Han	124	50.8	105	57.1	Reference	
Zhuang	120	49.2	79	42.9	0.99 (0.55–1.78)	0.98
HBsAg						
Negative	65	26.6	48	26.1	Reference	
Positive	179	73.4	136	73.9	1.19 (0.60–2.34)	0.61
anti-HCV						
Negative	217	88.9	161	87.5	Reference	
Positive	27	11.1	23	12.5	1.25 (0.51–3.09)	0.62
Smoking status						
No	181	74.2	134	72.8	Reference	
Yes	63	25.8	50	27.2	0.48 (0.12–1.85)	0.28
Drinking status						
No	174	71.3	130	70.7	Reference	
Yes	70	28.7	54	29.3	2.61 (0.69–9.89)	0.27
AFP (ng/mL)						
≤ 20	82	33.6	72	39.1	Reference	
> 20	162	66.4	112	60.9	1.00 (0.55–1.82)	0.99
Liver cirrhosis						
No	58	23.8	46	25.0	Reference	
Yes	186	76.2	138	75.0	0.84 (0.42–1.69)	0.63
BCLC stage						
A	113	46.3	54	29.3	Reference	
B	69	28.3	52	28.3	1.27 (0.61–2.61)	0.52
C	62	25.4	78	42.4	2.09 (1.04–4.24)	0.04
Tumor size						
≤ 3 cm	197	80.7	14	7.6	Reference	

	AFB1 (–)		AFB1 (+)			
Variables	**n**	**%**	**n**	**%**	**OR (95% CI)**	P_{trend}
> 3 cm	47	19.3	170	92.4	69.06 (33.62–141.86)	9.36×10^{-31}
MVD						
Negative	118	48.4	74	40.2	Reference	
Positive	126	51.6	110	59.8	2.56 (1.36–4.81)	3.46×10^{-3}
ES grade						
Low	129	52.9	97	52.7	Reference	
High	115	47.1	87	47.3	1.52 (0.64–2.07)	0.64

Abbreviations: AFP, α-fetoprotein; BCLC, the Barcelona Clinic Liver Cancer staging system; ES, Edmondson and Steiner grading system; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; MVD, microvessel density.

Table 2. The association between AFB1 exposure and clinic-pathological features of hepatocellular carcinoma cases.

all cases with hepatocarcinoma. According to the results from the CBX4 expression in cancerous tissues based on a large sample, IRS > 4 was regarded as positive CBX4 status. **Table 3** summarized the association between CBX4 expression in the cancerous tissues and the clinicopathological features, and results from multivariable logistic regression models proved that the levels of CBX4 expression were significantly related to increasing risk of liver cirrhosis (OR = 1.75 and 95% CI = 1.07–2.88), higher tumor stage (OR = 2.02 and 95% CI = 1.23–3.33), and increasing MVD (OR = 2.66 and 95% CI = 1.74–4.07). However, CBX4 expression levels did not affect other clinicopathological features such as tumor size, grade, AFP, and so on.

Results from Kaplan–Meier survival analyses further displayed that HCC patients with positive status of CBX4 protein expression had short MST [22.00 (18.00–26.00) months] and MRT [16.00 (10.88–21.12) months] compared with those with negative-status CBX4 protein [69.00 (52.75–85.25) months for MST and 48.00 (23.69–72.31) months for MRT, respectively] (**Figure 1B**). Taken together, CBX4 expression in the cancerous might be an important biomarker for HCC prognosis.

3.4. The joint effects of AFB1 exposure and CBX4 expression on HCC prognosis

Given that both AFB1 exposure and CBX4 expression modified HCC outcome, we questioned whether CBX4 expression interacted with AFB1 expression, and whether this interaction affected the prognosis of hepatocarcinoma. First, we analyzed the joint effects of AFB1 exposure and CBX4 expression on the prognosis of patients with HCC using Kaplan–Meier survival model (**Figure 2**). In this model, the combination of AFB1 exposure and CBX4 expression was divided into four groups: cases with negative-AFB1 and negative-CBX4 status (AC-1), cases with negative-AFB1 and positive-CBX4 status (AC-2), cases with positive-AFB1 and negative-CBX4 status (AC-3), and cases with positive-AFB1 and positive-CBX4 status (AC-4). We found MST and MRT gradually decreased from AC-1 to AC-4 (89.00–11.00 months for MST and more than 125.00–7.00 months for MRT, respectively) (**Figure 2A** and **B**).

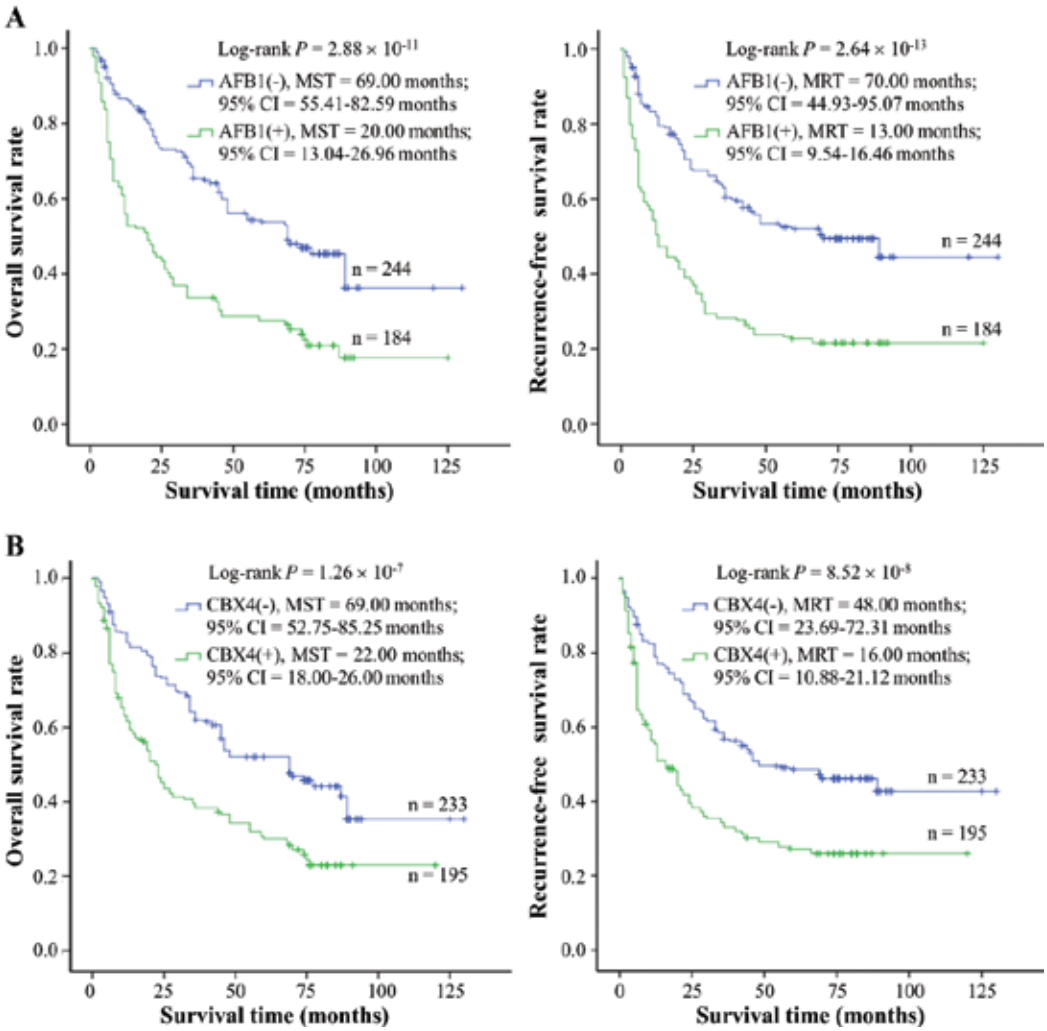

Figure 1. Both AFB1 exposure and CBX4 expression significantly correlating with hepatocellular carcinoma. AFB1 exposure levels were elucidated using the amount of AFB1-DNA adducts in the cancerous tissues. The CBX4 expression in cancerous tissues from 428 patients with hepatocellular carcinoma was tested using immunohistochemistry technique based on immunoreactive score system (IRS). To analyze, the levels of CBX4 expression were divided into two groups: Negative group (IRS ≤ 4) and positive group (IRS > 4). AFB1 exposure (A) and CBX4 expression (B) are associated with overall survival (left) and tumor recurrence-free survival (right) of hepatocellular carcinoma. Cumulative hazard function was plotted by Kaplan–Meier's methodology, and *P* value was calculated with two-sided log-rank tests. ***Abbreviations:*** CBX4, chromobox 4; MST, median overall survival time; MRT, median tumor recurrence-free survival time; CI, confidence interval.

We next finished multivariable Cox regression analyses based on the retread method with likelihood ratio test (including significant variables and all kinds of possible interactive variables) (**Table 4**), and found both AFB1 exposure and CBX4 expression in the cancerous tissues were independent prognostic factors. Furthermore, we also observed that AFB1 exposure significantly and multiplicatively interacted with CBX4 protein expression (interactive values, 1.98 for overall survival and 1.94 for tumor reoccurrence-free survival, respectively).

	CBX4 (–)		CBX4 (+)			
Variables	n	%	n	%	OR (95% CI)	P_{trend}
Total	233	100.0	195	100.0	—	—
Age (years)						
≤ 48	136	58.4	98	50.3	Reference	
> 48	97	41.6	97	49.7	1.35 (0.89–2.05)	0.67
Sex						
Man	160	68.7	130	66.7	Reference	
Female	73	31.3	65	33.3	1.14 (0.73–1.78)	0.57
Ethnicity						
Han	121	51.9	108	55.4	Reference	
Zhuang	112	48.1	87	44.6	0.96 (0.63–1.46)	0.85
HBsAg						
Negative	65	27.9	48	24.6	Reference	
Positive	168	72.1	148	75.9	1.17 (0.73–1.89)	0.51
anti-HCV						
Negative	209	89.7	169	86.7	Reference	
Positive	24	10.3	26	13.3	1.43 (0.74–2.75)	0.29
Smoking status						
No	176	75.5	139	71.3	Reference	
Yes	57	24.5	56	28.7	1.04 (0.37–2.93)	0.94
Drinking status						
No	172	73.8	132	67.7	Reference	
Yes	61	26.2	63	32.3	1.40 (0.51–3.83)	0.51
AFP (ng/mL)						
≤ 20	84	36.1	70	35.9	Reference	
> 20	149	63.9	125	64.1	1.11 (0.72–1.70)	0.64
Liver cirrhosis						
No	69	29.6	35	17.9	Reference	
Yes	164	70.4	160	82.1	1.75 (1.07–2.88)	0.03
BCLC stage						
A	112	48.1	55	28.2	Reference	
B	58	24.9	63	32.3	1.94 (1.16–3.24)	0.01
C	63	27.0	77	39.5	2.02 (1.23–3.33)	5.79×10^{-3}
Tumor size						
≤ 3 cm	121	51.9	90	46.2	Reference	
> 3 cm	112	48.1	105	53.8	1.24 (0.81–1.88)	0.33

	CBX4 (–)		CBX4 (+)			
MVD						
Negative	131	56.2	61	31.3	Reference	
Positive	102	43.8	134	68.7	2.66 (1.74–4.07)	6.65×10^{-6}
ES grade						
Low	132	56.7	94	48.2	Reference	
High	101	43.3	101	51.8	1.39 (0.92–2.11)	0.12

Abbreviations: AFP, α-fetoprotein; BCLC, the Barcelona Clinic Liver Cancer staging system; CBX4, chromobox 4; ES, Edmondson and Steiner grading system; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; MVD, microvessel density.

Table 3. The correlation between CBX4 expression and clinical pathological features of hepatocellular carcinoma.

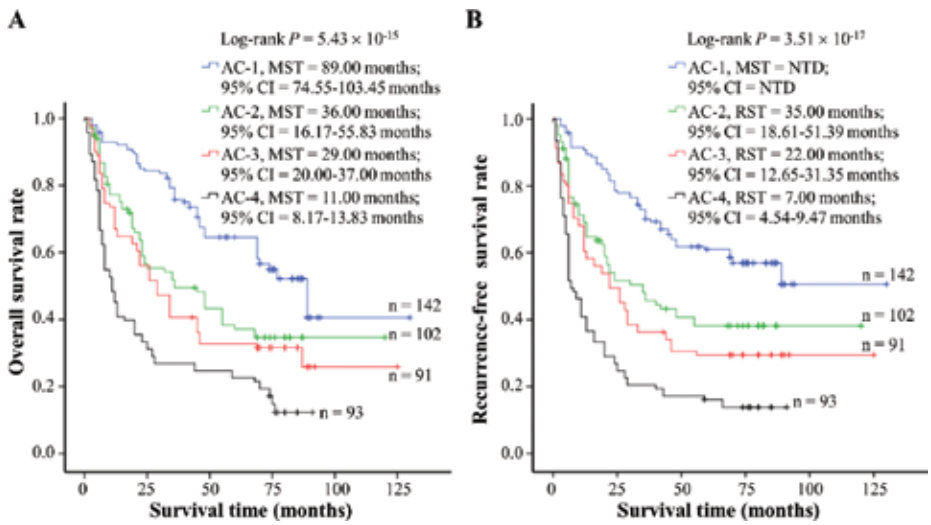

Figure 2. Survival analysis of CBX4 expression binding AFB1 exposure levels. The combination of CBX4 expression and AFB1 exposure was divided into 4 strata: Cases with negative-AFB1 and negative-CBX4 status (AC-1), cases with negative-AFB1 and positive-CBX4 status (AC-2), cases with positive-AFB1 and negative-CBX4 status (AC-3), and cases with positive-AFB1 and positive-CBX4 status (AC-4). This kind of joint analyses showed that interactive effects on the overall survival (A) and tumor recurrence-free survival (B) of patients with hepatocarcinoma. Cumulative hazard function was plotted by Kaplan–Meier's methodology, and *P* value was calculated with two-sided log-rank tests. ***Abbreviations:*** CBX4, chromobox 4; MST, median overall survival time; MRT, median tumor recurrence-free survival time; CI, confidence interval.

4. Discussion

In Guangxi Zhuang Autonomous Region, HCC is the most malignant disease. In the past decades, the annual incidence and death rate (AIR and ADR) of hepatocarcinoma in this area has been reported to remarkably increase (up to about 100–200 per 10,000 for AIR about 50 per 10,000 for ADR) [1]. Lots of epidemiological studies have shown that AFB1 exposure is

	OS		RFS	
Variables	HR (95%CI)	Ptrend	HR (95%CI)	Ptrend
AFB1	2.09 (1.64–2.65)	2.34×10^{-9}	2.29 (1.79–2.93)	3.82×10^{-11}
CBX4	1.76 (1.38–2.24)	4.66×10^{-6}	1.80 (1.41–2.30)	3.12×10^{-6}
AFB1 × CBX4	1.98 (1.61–2.59)	9.43×10^{-7}	1.94 (1.58–2.54)	8.17×10^{-5}

HR and corresponding 95% CI was calculated using multivariable Cox regression model (with retread method based on likelihood ratio test). ***Abbreviations:*** AFB1, aflatoxin B1; CBX4, chromobox 4; OS, overall survival; RFS, tumor reoccurrence-free survival; HR, hazard ratio; CI, confidence interval.

Table 4. The effects of AFB1 and CBX4 expression on the prognosis of cases with hepatocellular carcinoma.

the most important cause for this high AIR and ADR [1]. AFB1 is a known I-type chemical carcinogen produced by *Aspergillus parasiticus* and *Aspergillus flavus*, and has been proved to involve in the carcinogenesis and progression of HCC [4–10]. This carcinogenicity of AFB1 mainly results from its metabolic product binding to DNA and inducing DNA damage. Among DNA damage types induced by AFB1, AFB1-DNA adducts are very important, because of its non-enzymatic, time-dependent, and apparent persistent characteristics in the genomic DNA strands [3, 35]. Our previous studies have exhibited that AFB1-DNA adducts, especially from liver tissues, are highly associated not only with increasing HCC risk, but with the poor prognosis of HCC [2, 31, 36–39]. Here, our data displayed that increasing levels of AFB1 exposure significantly correlated with higher tumor stage, increasing tumor size, and higher MVD; furthermore, AFB1 was also poor prognostic marker for HCC. Taken together, these data suggest that AFB1 may involve in the startup and progression of HCC.

Because several previous studies have exhibited that CBX4 can progress tumorigenesis via several signal pathways, including CBX4/HIF-1α/VEGF pathway [20, 25, 26, 34], CBX4/HDAC3/Runx2 pathway [21], CBX4/P63 pathway [22], CBX4/miR-195 pathway [40], CBX4/CtIP pathway [41], and CBX4/P53 pathway [42, 43], here we investigated the effects of CXB4 expression on HCC outcome. We not only found that increasing CBX4 expression in the cancerous tissues is a poor prognostic biomarker for HCC, but this increasing expression is associated with clinicopathological features such as tumor size, tumor stage, and angiogenesis. Supporting our findings, several recent reports further prove that CBX4 can govern the several biofunctions of HCC, including proliferation, invasion and metastasis, angiogenesis, and metastasis [20, 25, 26, 34, 40, 44].

Noticeably, some evidence of the joint effects of CBX4 and AFB1 on HCC outcome was observed in the prognostic analyses based on the gene-environmental joint effects. Our results showed that CBX4 expression significantly and multiplicatively interacted with AFB1 exposure levels, and that this multiplicative interaction remarkably increased the death risk and tumor reoccurrence risk of patients with HCC. Recently, two studies from high AFB1 exposure areas have also reported that the dysregulation of CBX4 in the cancerous tissues from patients with hepatocarcinoma increases MVD, promotes angiogenesis, and increases sensitivity of HCC cells on anti-cancer drugs [33, 34]. Altogether, these results are indicative of the angiogenesis induced by CBX4 involving in the progression of AFB1-related HCC.

In summary, our present study proposes that CBX4 expression in the cancerous tissues can act as a valuable biomarker for AFB1-related HCC. However, several limitations confine the value of this study. First, because of the hospital-based retrospective design, selective bias may take place. Second, because liver damage itself affects AFB1 metabolite and may increase the amount of AFB1-DNA adducts, the prognostic and interactive values of AFB1 and CBX4 may be underestimated. Finally, we did not do functional and mechanical analyses. Therefore, detailed functional analyses deserve further evaluation on the basis of the foresighted design and the combination of AFB1 and CBX4.

Conflicts of interest and source of funding

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Abbreviations

AFB1	aflatoxin B1
AFBEX	AFB1–8,9-exo-epoxide
AFBEN	AFB1–8,9-endo-epoxide
BCLC	The Barcelona Clinic Liver Cancer
CBX4	chromobox 4
CI	confidence interval
CYP	cytochrome P450
ES	The Edmondson and Steiner
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus

HBsAg	hepatitis B surface antigen
IRS	immunoreactive score system
MRT	median tumor reoccurrence-free survival time
MST	median overall survival time
MVD	microvessel density
OD	odd ratio.

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Cancer is a DNA disease in which the early stage is represented by the inactivation of suppressor genes and activation of oncogenes, which result in transformed cells that grow out of biological control. Tumor progression is locally favored by mitogenic effects of hormones, or growth factors that stimulate the tumor's growth, or by inducing angiogenesis. The book contains chapters written by experts in the topic, and exhibits current developments in the methodology of cell and molecular biology, which have deeply advanced the understanding of cancer's prevention and prognosis. We hope that it will be helpful for physicians, researchers, and students in life sciences, and will stimulate discussion and research for new therapeutic approaches against cancer.

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