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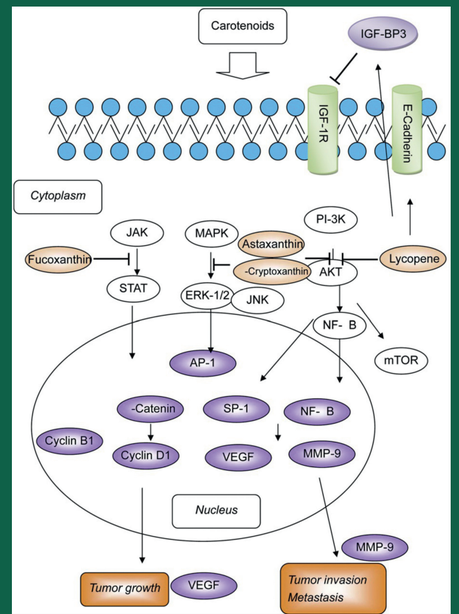

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Editorial

Humans and cancer: An ongoing fight

Cancer is one of the leading causes of death worldwide. Findings from laboratory and clinical researches conducted during the last few decades have made a substantial contribution to the development of more effective diagnostic and surgical techniques, pharmacological regimens, and therapeutic protocols. In this issue of *BioMedicine*, we display several review articles, a clinical study, and a clinical spotlight that focuses on carcinogenesis.

Hepatitis C, a chronic disease caused by infection with the hepatitis C virus (HCV), is endemic in many Asian countries. Epidemiological investigations have shown that chronic HCV infection is associated with the development of liver cancer and that it is highly associated with the degree of morbidity in patients with hepatocellular carcinoma. One of the studies in this issue focuses on seromarkers that are highly predictive of various HCV-related diseases. Lee et al conducted a 15-year follow-up study of the outcomes of 1095 patients who were seropositive for anti-HCV antibodies. The researchers found that, besides reflecting the risk of inducing hepatocellular carcinoma, anti-HCV seropositivity and elevated serum levels of HCV RNA also increased the risk of mortality due to extrahepatic diseases such as cerebrovascular disease and renal disease. The authors conclude that both anti-HCV seropositivity and HCV RNA levels are crucial factors for the deterioration of renal and brain functions in infected hosts.

The results from numerous experiments indicate that cancer stem cells as well as the up-regulation of integrin, matrix metalloproteinases, endothelial growth factor, fibronectin, transforming growth factor-β1, and intercellular adhesion molecule-1 expression in tumors favor cancer cell migration and invasion. Thus, targeting cancer stem cells, associated molecules, and their related pathways may enhance the possibility of mitigating the development or progression of cancer. These findings also imply that monitoring the variation of certain biomarkers can help in the evaluation of cancer progression.

Traditional Chinese Medicine is widely used as an alternative to conventional cancer therapies because the majority of the regimens demonstrate low levels of toxicity, have very few side effects, and are less expensive to administer than chemotherapy and radiation therapy. Anthraquinone and its derivatives, namely aloe-emodin, danthron, emodin, chrysophanol, physcion, and rhein, have been shown to have potential anticancer properties. Aloe-emodin in particular has attracted much attention because it has been shown to inhibit angiogenesis, invasion, migration, chemical-induced carcinogen-DNA adduct formation, and the expression of HER2/neu, CKII kinase, and p34cdc2 kinase in human cancer cells. In addition, carotenoids have been shown to have anticancer effects by interrupting various stages of carcinogenesis such as initiation, promotion, progression, and metastasis. Therefore, dietary or supplemental intake of carotenoids or foods rich in these compounds may prevent the development of cancers.

Although much progress in the fight against cancer has been made during the past few years, the disease is still far from being conquered. More efforts from multiple directions are required to update our understanding regarding the pathological characteristics and mechanisms of cancer development.

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Review article

Role of cancer stem cells in brain tumors

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ABSTRACT

Cancer stem cells contribute to tumor progression, resulting in their capacity to persistently self-renew and propagate tumors. Recent evidence suggests that brain cancer stem cells (BCSCs) are critical for tumor vascular development and therapeutic resistance. Here, we outline the crucial molecular mechanisms and interacting niches involved in BCSCs, which uncovering multiple potential targets for malignant brain tumors and may provide clues for developing novel antibrain tumor treatments.

1. Introduction

Both adults and children can experience malignant brain tumors, e.g., glioma, medulloblastoma, and ependymoma, yet current translational medicine has not resulted in significant improvement in survival. Glioblastoma (GBM) is the most frequent adult primary brain tumor and has an extremely poor outcome with only a median survival of 15 months [1]. Brain tumors, predominantly medulloblastomas, are comprised of a heterogeneous group of tumors and also a leading cause of cancer death in children. Even though therapies for primary tumor response have improved, these malignancies recur most of the time, and newer treatment modalities are urgently needed to target brain tumors. Based on extensive studies of brain tumors, it appears that targeting the regulatory signaling pathways, tumor microenvironment, and characterized stem cells form the basis for future development of targeted therapies [2–4]. Brain cancer stem cells (BCSCs) or brain tumor initiating cells (BTICs) belong to a sub-population of cells that possesses capacity for self-renewal, multipotency, and tumor

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propagation and has been attributed to increased angiogenesis and drug resistance [5–13]. There is mounting evidence that unveiled the molecular actions of BCSCs, leading to a significant number of potential targets. In this review, we will discuss the role of BCSCs in initiation and propagation in brain tumor as well as how to targeting brain tumor by directly or indirectly inhibiting BCSCs. Moreover, we will also highlight the niche of BCSCs, particularly in neurovascular interactions, as target for brain tumor therapies.

2. Cancer stem cells in brain tumor

2.1. *Characteristics and stem cells for malignant brain tumor*

Primary brain tumors (PBT) contain malignant heterogeneous groups that originate and arise from the brain and the central nervous system (CNS). According to the World Health Organization classification, the most prevalent PBT in elderly is gliomas, and the most malignant type of gliomas is grade IV, also known as glioblastoma multiforme (GBM) [14]. Both aggressive primary GBM and secondary glioblastoma multiforme as a result of a low-grade glioma progressing into highly malignance have very poor prognosis even with radiotherapy and chemotherapy [15]. More importantly, GBM possesses tissue-scattering distribution pattern accompanied by extensive diffusion within the brain, making it difficult for surgical resection [16]. In children, the highest incidence of brain tumor is medulloblastomas, which is commonly developed from the malignant transformation of progenitors of the external granular layer in the cerebellum, and is by far the most aggressive form of pediatric cancer with poor outcome. Compared with adult glioma, medulloblastoma is thought to develop from an embryonal tumor and shown to express several genes which involved in differentiation of neural stem cells, including Sox2, Bmi1, and Musashi 1[17,18].

The concept of cancer stem cells (CSCs) was first hypothesized in the studies of acute myeloid leukemia [19,20] and subsequently found in solid tumors, including brain tumors. Several groups have identified and characterized CSCs in clinical samples from patients with glioma and medulloblastoma [5,17,21–23]. Stem cells possess multipotent capacity to generate different types of mature cells in the tissue origin. Importantly, the key property of stem cells is the ability to self-renew to maintain a constant cell number in adult tissues throughout life. Stem cells acquire self-renewal capacity by executing asymmetric divisions to reliably reserve a copy of the mother cells, while producing mature progenitor simultaneously. Cancer stem cells have been proposed to arise from mutation in normal stem cells, and subsequently grow and differentiate to generate primary tumors. Similar to normal stem cells, cancer stem cells are able to self-renew, develop heterogeneous populations of daughter cells, and proliferate extensively [24].

2.2. *Enrichment of BCSCs*

Isolation CNS cancer stem cells in specimens by purification of $CD133^+$ cells from human glioblastoma and medulloblastoma allows generation of neurospheres and growth of tumor stem cell populations [23,25]. Neurospheres can be repeatedly separated into single cells, and these single cells can produce a new neurosphere. Evidence of self-renew is commonly observed from the ability of single cells to repeatedly produce neurospheres [26]. Additionally, as few as 100 $CD133^+$ human glioma cells which were transplanted into the brain of severely immunodeficient (NOD-SCID) mice developed gliomas, whereas no tumors formed from transplantation of 10^5 $CD133^-$ cells from the same tumor [5]. Subsequent studies revealed controversial findings that identified glioma stem cells as CD133 negative [27,28]. This controversy might be caused by the use of distinct methods and techniques to detect CD133 and factors that are able to affect its detection [28]. Albeit the contradiction, CD133 continues to be a frequently used marker for BCSCs, followed by several other BSCS markers, such as A2B5 [29], stage-specific embryonic antigen (SSEA-1/CD15) [30], L1CAM (CD171) [31], aldehyde dehydrogenase 1 (ALDH1) [32], integrin α6 (CD49f) [33], CD44 [34], and epidermal growth factor receptor (EGFR) [35].

Recently, Clement and colleagues [36] attempted an alternative method by utilizing intrinsic autofluoresce properties and distinct morphology to isolate human glioma stem cells without using molecular markers. For example, subpopulations of human gliomas with tumor initiating activities were identified by autofluorescence emission at 520 nm after excitation at 488 nm. Because the differences in marker expression or enrichment capacity of BCSCs varies from one laboratory to another, it is important to standardize the method of cell sorting by flow cytometric analysis for easier comparison of methods and data from different groups [37].

3. Niche of BCSCs

Gliomas appear to be highly vascular, and endothelial cells, pericytes, and astrocytes have been shown to serve as the functional unit for neurovasculature to foster tumor growth. Staining of BCSC markers and tumor vasculature from glioblastoma specimens showed a physically colocalized pattern, which appears in the angiogenic regions within glioblastoma [2,9]. Neural stem cells (NSCs) also share a defined vascular niche with medulloblastoma CSCs [9,38,39]. BCSCs are found adjacent to the neurovasculature in brain tumors, suggesting that the existence of molecular signaling and microenvironmental factors in the specialized perivascular niche make significant contributions to maintain BCSCs. Additionally, self-renew and proliferation of BCSCs can be promoted by tumor endothelial cells such that simultaneous injection of both CSCs and endothelial cells accelerates tumor initiation and progression [40], indicating that cell-to-cell signaling within perivascular niche is important to brain tumor development.

The maintenance of NSCs depends on their interactions with the extracellular matrix (ECM) [41], implying that ECM has a vital role in perivascular niche to regulate the maintenance of BCSCs. Although the components of the ECM have not been defined in gliomas perivascular, different groups have reported the expression of several laminin chains, including α2, α3, α4, α5, α3β1, and γ1, in brain tumors. Ljubimova and colleagues [42,43] also reported that expression of laminin depends on the tumor grade and is associated with

patient survival. More importantly, receptor integrin-α6 is highly expressed in BCSCs, and integrin-α6 is able to recognize several forms of laminin [41]. The interaction between integrin-α6-positive BCSCs and laminin in the perivascular niche may promote BCSC maintenance. Alternatively, the survival and tumorigenic ability of BCSCs were decreased by targeting integrin-α6 via lentiviral delivered short hairpin RNA (shRNA) [33]. Furthermore, ECM components that are located in the perivascular are capable of accelerating the BCSCs phenotype. More in-depth mechanisms remain to be identified and characterized.

In the paracrine regulatory pathway that associates endothelial cells with BCSCs functions is mediated by nitric oxide (NO). Accumulated evidence indicates that NO enhances tumorigenesis and gives rise to increased levels of endothelial NO synthase (eNOS) in gliomas [44]. The perivascular NO produced by eNOS has been suggested to facilitate glioma progression in a glioblastoma mouse model [45]. Thus, BCSCs may support their survival through mechanisms similar to autocrine regulatory pathway. For instance, Eyler and colleagues [46] demonstrated that inducible NO synthase (iNOS)-generated NO in BCSCs promotes glioma growth in xenograft mouse model. These findings suggest that NO comes from the endothelial cells or BCSCs and is a critical factor involved in modulation of BCSC maintenance.

4. Molecular signaling of BCSCs

A crucial issue in CSC biology is to delineate the regulatory signaling pathways that are involved in maintaining their phenotypes. Glioma CSCs were earlier reported among solid tumor CSCs and appear cellular hierarchy to initiate tumor formation [5]. Notably, glioma CSCs have been shown to promote tumor angiogenesis and are also highly resistant to chemotherapy and radiotherapy [5–7,13], and, thus, raise the importance of elucidating the underlying molecular mechanisms underlying in the regulation of BCSCs to develop more efficient therapies against BCSCs.

4.1. *Signaling of development and growth factor*

External signals from the microenvironment such as stromal, immune response, and other non-stem tumor cells persistently influence CSC actions. Thus, cell surface ligand-receptor systems also play an important role in the regulation of CSCs by extracellular and paracrine signals. There is accumulating evidence to suggest that receptor-mediated pathways control the physiologic functions of BCSCs.

4.1.1. *Notch signaling*

In both invertebrates and vertebrates, Notch signaling is crucial for regulating cell fate determination in many cell lineages through cell-cell communication. Notch proteins are transmembrane receptors, and their intracellular domains (ICD) can be cleaved by the γ-secretase complex for translocation into the nucleus to function as a transcriptional factor upon ligand binding. The significance of Notch signaling pathway has also demonstrated its high conservation during evolution. Notch can facilitate normal NSC proliferation that results in repression of their differentiation [47,48]. Notch has been implicated in brain tumor based on a significantly correlation observed between Notch-1 expression and its ligands, such as Delta-like-1 and Jagged-1 in high-grade gliomas and medulloblastomas [49,50]. Previous studies have indicated that Notch signaling potentially regulates BCSCs in medulloblastomas. Moreover, elevated expression of Notch in BCSCs has been shown to augment the sensitivity to inhibitors of the Notch pathway [51]. Notch proteins are also associated with CSCs, which can enhance the stem cell marker, Nestin in gliomas. The activation of Notch and K-Ras in mouse glioblastoma model yielded proliferative lesions that are located in NSC-occupied subventricular zone (SVZ) due to increased expression of Nestin and glioma formation [52]. Moreover, increased neurosphere-like colonies are also observed while Notch signaling is activated in glioma cell lines [53].

4.1.2. *Hedgehog signaling*

The Hedgehog pathway plays a key role in the regulation of embryogenesis, CNS development, and neural stem cell proliferation and differentiation [54,55]. Upon Hedgehog ligand binding to their receptors, Gli transcription factors and glioma-associated oncogene are activated and subsequently translocated into nucleus to turn on or off their target genes. Aberrant Hedgehog pathways are correlated with primary brain tumor, such as medulloblastoma and evaluated by genetic medulloblastoma models [56]. Moreover, Hedgehog signaling enhances CSCs self-renew and tumorigenicity in gliomas [57]. Increased glioma apoptosis was observed when treated with Hedgehog inhibitor cyclonamine or transduced interference RNA of Gli, which inhibits proliferation and self-renewal of glioma CSCs. Importantly, combining Hedgehog inhibitor and traditional chemotherapy agent, such as temozolomide (TMZ) for treating GBM, has been shown to improve cell death of BCSCs cell death and reduce tumor cell proliferation [57]. Several reports also demonstrated that cyclonamine treatment not only abolishes BCSCs resulting in the failure of tumor progression *in vivo* but also increases the sensitivity of BCSCs to radiation therapy [58]. Collectively, these findings indicate that the Hedgehog pathway is critical for BCSCs, and inhibitors that target this pathway may enhance the efficacy of standard treatment for brain tumor.

4.1.3. *Receptor tyrosine kinase*

The receptor tyrosine kinase (RTK) family is critically involved in growth factor-mediated oncogenesis, and among them, one of the first identified and best-characterized RTK in glioma is EGFR. Enhancement of EGFR signaling pathway is frequently observed in malignant glioma cells such as the constitutively active EGFR type III variant (EGFRvIII) and aberrantly amplified copy number of EGFR. In addition, glioma-like lesions are formed after transduction of Nestin-positive neural stem cells with EGFRvIII in orthotopic mouse model [59]. Consistent of this notion, Li and colleagues [60] revealed that transduction of constitutive EGFRvIII into phosphatase and tensin homolog deleted on chromosome 10-deficient neural precursor cells is sufficient to generate glioblastoma in which the transformed cells harbor tumor and stem-cell marker, CD133, and possess self-renewal ability. Additionally, EGF can promote formation of spheres and facilitate the self-renewal ability in $CD133^{+}$

sub-populations derived from three brain tumor patients [61]. Interestingly, gefitinib, which is a selective inhibitor of EGFR, induced apoptosis and significantly repressed $CD133^+$ BCSCs [61]. Together, these data suggest an important role for EGFR signaling pathways in glioma and BCSC biology.

RTK signaling can be propagated and amplified by the downstream cascades, including the serine/threonine specific protein kinase B (AKT)/phosphoinositide 3-hydroxykinase (PI3K) pathway. Upon RTK activation, AKT enhances cell survival, proliferation, and invasion. It has been demonstrated that glioma CSCs rely more on the AKT signaling pathway than the paired non-stem glioma cells. AKT inhibitors have been shown to reduce the number of viable BCSCs and glioma neurospheres, and attenuate intracranial tumor formation [62]. Collectively, targeting BCSCs subpopulation by inhibition of AKT via specific inhibitors can suppress brain tumor malignancy.

4.1.4. *Bone morphogenetic protein*

The major role of bone morphogenetic proteins (BMPs) is to mediate bone and cartilage development [63]. BMPs bind to the BMP receptors, which are transmembrane serine/threonine kinases, and subsequently activate the canonical regulatory proteins, Smads (Smad1/5/8). Phosphorylated Smads then interact with co-activator Smad4 to form a protein complex that translocates to the nucleus where it regulates transcription and gene expression. BMP signaling regulates NSC proliferation and apoptosis and mainly facilitates the differentiation of NSCs [64]. Interestingly, BMPs have also been shown to suppress the stem-like and cancer stem cell precursors of human glioblastomas [65]. BMP ligands can abolish BCSCs population by inducing stem cell to undergo differentiation into astroglial and neuron-like cells [65]. Glioblastoma treated with BMPs *in vivo* can effectively delay tumor growth and invasion [65]. These results offer a new therapeutic approach to treat glioblastoma by inducing BCSC differentiation rather than directly killing them.

Lee and colleagues [66] also demonstrated that BMPs could induce BCSC differentiation. Interestingly, they found that BMPs actually facilitate BCSCs proliferation rather than differentiation by EZH2-dependent epigenetic silencing of the BMP receptor 1B (BMPR1B) similar to early embryonic NSCs. However, enforcing the expression of BMPR1B can sensitize BCSCs to BMP-mediated differentiation. Therefore, an individual's epigenetic features may affect the response of BCSCs treatment.

4.2. *Epigenetic signaling*

Epigenetic gene regulation has a pivotal role in regulation of embryogenesis, stem cells, and human cancers. In cancers, aberrant epigenetic modulation is correlated with chromatin regulation of gene expression that maintains the embryonic stem cell (ESC) or progenitor cell state. Accumulated data suggest that cancer stem cells have the gene expression signature reminiscent of ESCs and that BCSCs are epigenetically deregulated.

4.2.1. *Bmi1*

The polycomb group (PcG) protein Bmi1 is an epigenetic silencer and has been implicated not only in the regulation stem cells in multiple tissues but also in mediating self-renewal of stem cells. Postnatal brain, and human brain tumor samples extensively expressed Bmi1, and defective stem cell compartments in the brain were observed in Bmi1-deficient mice [67]. Bmi1 also possesses oncogenic ability involved in several types of cancer, including glioblastoma. Bruggeman and colleagues [68] demonstrated that Bmi1 is not only required for astrocytes transformation and differentiation *in vitro* and *in vivo* but also essential for neural stem cells transformation and differentiation ability. Moreover, Bmi1 knockout neural stem cells can develop into low-grade tumor compared to wild type and are able to progress into high-grade gliomas. Fewer NSCs in Bmi1-deficient tumors express the stem cell marker, Nestin, indicating that there might be less number of BCSCs compared with the control. The repression of neurogenic capacity was observed both in transformed and non-transformed Bmi1-deficient glial cells, implicating Bmi1 as a key mediator involved in controlling NSC and CSC differentiation.

Consistently, there is direct evidence that shows Bmi1is highly expressed in enriched $CD133^+$ cells in human GBM [68]. Depletion of Bmi1 expression in GBM cell lines inhibited neurosphere and clonogenic formation. Furthermore, knocking down Bmi1 strongly inhibited brain tumor development even with to 1×10^5 cells inoculated in NOD/SCID mice. Gene expression profiling indicated that Bmi1 attenuates alternate tumor suppressor signaling pathway that can be activated to compensate for the deletion of INK4A/ARF, a inhibitor for cell cycle by arresting cell in G1 phase, and activation of AKT/PI3K. Disruption of INK4A/ARF, which is a tumor suppressor gene to regulate RB and p53 pathways, is one of the most common mutations existing in human GBM [69,70]. Also, the activity of AKT/PI3K is extremely increased in GBM and treatment of an AKT inhibitor enhances the sensitivity against CSCs due to decrease GBM malignancy [62]. These results support Bmi1 as an important in sustaining cancer stem cell renewal in human GBM.

4.2.2. *EZH2*

Enhancer of zeste homolog 2 (EZH2) is a catalytically active component of polycomb repressive complex 2 (PRC2) and participates in transcriptional silencing of specific genes via trimethylation of histone 3 at lysine 27 (H3K27me3). Induction of EZH2 is not only involved in hematopoietic and solid tumor progression but also associated with poor prognosis. Additionally, EZH2 plays a key role in stem cell maintenance, differentiation, and self-renew during development. Suva and colleagues [71] demonstrated that EZH2 is overexpressed and enriched in glioblastoma CSCs. The self-renewal and tumor initiating abilities of glioblastoma CSCs *in vivo* are dramatically inhibited when pharmacologic inhibitors or shRNAs are used to target EZH2 [71]. More recent studies also indicated that disruption of EZH2 reduces the expression of CD133 and proliferation of glioblastoma CSCs [72]. Together, these data support the potential of EZH2 as a valuable therapeutic target for GBM treatment.

4.2.3. *MicroRNAs*

MicroRNAs (miRNAs) are small noncoding RNAs that can modulate gene expression by targeting specific genes to silence protein expression. miRNAs play a significant role in cell fate determination and proliferation involved in

development and cancer biology. Several studies have been identified several miRNAs that specifically regulate brain development and neural differentiation through microarray analysis of miRNA expression in mammalian brain. For instance, the expression of miR-21 is significantly increased in glioblastomas, and attenuated its action triggers caspase dependent apoptosis [73]. Similar to miR-21, a recent study indicated that the miR-17-92 cluster is highly expressed in primary astrocytic gliomas and glioblastomas compared with the normal brain, and it is also implicated in the progression of low-grade to more aggressive brain tumors. Inhibitors of miR-17-92 were shown to suppress glioblastoma spheroids by promoting apoptosis and reducing proliferation [74].

Silber and colleagues [75] investigated the possible role of miRNA in the regulation of CSCs in glioma. Specifically, miR-124 and miR137 expression levels were dramatically reduced in anaplastic astrocytomas (grade III) and GBM (grade IV) compared with normal brain tissues. Ectopic expression of miR-124 and miR137 in GBM cells can reduce cell proliferation and promote differentiation of glioma stem cells. These data indicate that these two miRNAs could serve as a tumor suppressor in BCSCs. In addition, another study demonstrated that the expression of miR-451 is lower in glioma $CD133^+$ CSCs compared to $CD133^-$ non-glioma stem cells [76], suggesting the possibility of inducing the expression of miR-451 to destroy neurosphere formation and reduce giloma CSCs proliferation.

4.3. *Signaling in CSC niches*

Several studies have reported that the interactions and regulatory signaling pathways between BCSCs and perivascular endothelial cells are important for brain tumor progression and clinical targeting. BCSCs can not only receive signals from microenvironment but also propagate signals to affect the environment. The perivascular niche of brain tumor is the best example for such communication.

4.3.1. *Vascular endothelial growth factor*

BCSCs are able to produce the well-characterized proangiogenic factor such as vascular endothelial growth factor (VEGF) [6,11]. A paracrine role of VEGF generated from BCSCs is demonstrated through the inhibition of endothelial cell proliferation and tube formation when BCSC-conditional medium is supplied with VEGF-neutralizing or VEGFR2-blocking antibodies (bevacizumab). The significant suppression of human glioma growth in xenograft mice is observed while treatment with anti-VEGF or anti-VEGFR2 antibodies, that resulting in a reduction of blood vessels density [6,11]. In addition to undoubtable contribution of targeting effects of VEGF on endothelial cells, glioma cells also express VEGF and VEGFRs [77]. VEGF-VEGFR autocrine signaling can enhance glioma cells proliferation and viability, and blockage of this effect leading to increase the response to radiation-induced cell death [77]. Together, these results support that glioma progression relies on BCSCs-driven generation of VEGF through both autocrine and paracrine signal.

4.3.2. *Hypoxia inducible factors*

Microenvironmental stress, including nonphysiologic levels of oxygen, pH, and metabolites, can influence tumor development through distinct signaling pathway. For example, low oxygen concentration and hypoxia are crucial to maintaining BCSCs via hypoxia inducible factors (HIFs), in particular, HIF2α [2]. Under hypoxia condition or overexpression of a non-degradable form of HIF2α promotes non-stem glioblastoma cells to gain self-renewal capacity that leads to cellular transformation [78]. Under hypoxia, studies have shown that the signature genes such as lysyl oxidase (LOX), VEGF, HIG2, and prominin1 (CD133) of glioblastoma stem cell are overexpressed under hypoxia condition [79,80]. Importantly, response to hypoxia in BCSCs can be attenuated by digoxin, a HIF inhibitor [80], suggesting that HIF proteins could potentially become therapeutic targets for malignant brain tumors.

5. Targeting of BCSCs

5.1. *Direct BCSC targeting*

The standard treatment for brain cancer commonly uses both irradiation and chemotherapy, e.g., temozolomide (TMZ). However, resistance to irradiation and TMZ often occurs due to the enrichment of $CD133^+$ fraction in tumor [7,13,81]. Therefore, novel treatments for brain cancer that block function of BCSCs could potentially overcome resistance to standard therapy. Direct targets of BCSCs include Notch, Hedgehog/Gli, EGF/EGFR/AKT pathway, Bmi1, EZH2, and miRNAs, all of which have been shown to sensitize BCSCs to drug treatments and inhibit BCSC survival [82]. Also, as mentioned previously, another strategy to enforce BCSC and reduce their tumorigenic potential is to induce BCSC differentiation, such as through BMP signaling [65]. For instance, BCSCs treated with BMP by implantation of BMP-bearing beads in glioblastoma mouse model significantly attenuated their transforming capacities [65].

5.2. *Indirect BCSC targeting*

Strategies that indirectly target BCSCs focus on the niche or cell microenvironment that harbors key determinants to sustain growth and survival of BCSCs. In perivascular niche, bevacizumab, a well-known inhibitor of VEGF, inhibited tumor vasculature, decreased $CD133^+$ BCSC number, and significantly reduced tumor size [9]. Moreover, there are compelling data demonstrating that the hypoxia microenvironment is a distinct niche that enriches BCSCs through upregulation of HIF2α. Downregulation of HIF2α can reduce stem cell marker expression, neurosphere formation, and VEGF signaling [10,82]. These landmark studies not only characterized the importance of perivascular or hypoxia niches for BCSCs but also identified new therapeutic approaches that target them.

6. Conclusion

In the past decade, CSC research has provided distinct new views in cancer biology. In particular, the cellular hierarchy and tumorigenic ability of BCSCs are highly attractive as targets for therapeutic development against brain cancer.

Moreover, by addressing the regulatory molecular mechanisms and interactions between BCSCs and the niches that maintain and propagate them, researchers have provided extraordinary insights on potential therapies that directly or indirectly target BCSCs. While extensive investigations have broadened the understanding of brain cancer biology, there is still a lack of substantial improvement in brain cancer patient survival. Therefore, there is an urgent need for more in-depth investigations to unravel the underlying molecular mechanisms that support and maintain BCSCs as well as the development of novel therapies against brain cancer.

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Review article

Molecular mechanisms of chondrosarcoma metastasis

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ABSTRACT

Chondrosarcoma is highly malignant, with a strong capacity for local invasion as well as distant metastasis. Surgical resection remains the primary mode of therapy. This cancer shows a predilection for metastasis to the lungs. This article will highlight numerous molecular mechanisms mediating cell motility, as described in such cases. Numerous experiments have demonstrated that upregulation of integrin and matrix metalloproteinases (MMPs) and intercellular adhesion molecule-1 (ICAM-1) expression lead to increased tumor cell migration and invasion. Data from these experiments suggest that targeting these pathways and molecules may enhance control of chondrosarcoma and decrease metastasis ratio.

1. Introduction

Chondrosarcoma is the second most common primary malignant bone tumor after osteosarcoma and most common in patients age 20 years or older [1]. It is a malignant bone tumor of chondrogenic origin, typically occurring in the fourth and fifth decades of life [2]. Chondrosarcoma can be divided into primary subtypes, based on histopathology: conventional, dedifferentiated, clear cell, and mesenchymal [3]. Local invasiveness and metastatic potential primarily depend on tumor grade and are primary predictors of clinical outcome [4]. Most diagnosed tumors (>90%) are of the conventional subtype, with approximately 90% of these showing pathology of low to intermediate grade (grades 1 and 2).

Chondrosarcomas primarily develop in the skull base in cartilaginous rests within the synchondroses of basilar skull bones [5]. These tumors are most commonly observed in the petrous portion of the temporal bone as well as in petro-occipital, spheno-occipital, and sphenopetrosal synchondroses areas. Location of these tumors highlights differences in bone development between the cranial vault and skull base. Bones of the cranial vault chiefly develop by intramembranous ossification; those in the skull base form by endochondral ossification and contain remaining chondrocytes, even in the mature skull [6]. Chondrosarcomas account for 6% of skull base tumors but for only 0.15% of all intracranial tumors. Most chondrosarcoma is characterized by low- and intermediate-grade conventional-type lesions with indolent growth and low metastatic potential. Only 12% of all skull base chondrosarcomas show mesenchymal type histopathology, associated with a higher grade and worse clinical prognosis [7]. Compared to chondrosarcomas with

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conventional histopathologic traits, those with mesenchymal characteristics are associated with an approximately tenfold increase in 5-year mortality.

Chondrosarcoma is relatively resistant to radiotherapy and chemotherapeutic regimens [8,9], making surgical resection the primary treatment, with an average 5-year survival rate of 50% [8,10]. Because chondrosarcoma is highly malignant, with a strong capacity of local invasion and distant metastasis [11,12], an approach in which ability of a tumor to invade and metastasize is decreased may facilitate development of effective adjuvant therapy. Numerous studies have investigated signaling pathways involved in chondrosarcoma growth and invasion. This article summarizes molecular mechanisms involved in chondrosarcoma metastasis.

2. Metastasis of chondrosarcoma

Carcinomas metastasize following a complex succession of cell-biologic events, collectively termed invasion-metastasis cascade, whereby epithelial cells in primary tumors invade locally through surrounding extracellular matrix (ECM) and stromal cell layers; intravasate into lumina of blood vessels; survive transport through the vasculature; arrest at distant organ sites; extravasate into parenchyma of distant tissue; initially survive in foreign microenvironments for micrometastases; and reinitiate their proliferative programs at these metastatic sites [13]. Many such complex cell-biologic events are orchestrated by molecular pathways operating within carcinoma cells. Notably, nonautonomous cell interactions between carcinoma and nonneoplastic stromal cells play vital roles throughout the invasion-metastasis cascade. Deregulating these intrinsic and extrinsic signaling cascades allows incipient metastatic carcinoma cells to generate high-grade, life-threatening malignancy [13].

Chondrosarcoma is a highly malignant tumor with the potent capacity to cause local invasion and distant metastasis, preferentially in the lungs [14]. Recurrence typically occurs as pulmonary metastases or, less frequently, metastases to distant bones or as a local recurrence. Initial steps in pulmonary metastases resemble those of metastases to any other site. Primary tumor cells invade the surrounding normal tissue by producing proteolytic enzymes that traverse walls of small blood vessels in normal tissue or by enzymes induced by a tumor that then enters the circulation [15]. These tumors cells then travel to distant organ sites. These events have been described as inefficient because many cancer cells do not survive action of normal protective host-surveillance mechanisms during these initial stages [16,17]. Surviving cancer cells can enter the lungs and cause pulmonary metastases. Malignant cells must possess specific properties for this to occur: e.g., capacity for migrating to lungs and generating their own blood supply (Fig. 1). Each step entails important molecular interaction between tumor cells and normal host cells, each a potential target for development of drugs designed to abrogate the metastatic process. This article highlights several molecular mechanisms mediating cell motility described for human chondrosarcoma. Numerous experiments have demonstrated that upregulation of integrin and matrix metalloproteinases (MMPs), and intercellular adhesion molecule-1 (ICAM-1) and cyclooxygenase-2 (COX-2) expression lead to increased tumor cell migration and invasion.

2.1. *Integrin and metastasis of chondrosarcoma*

Integrins are transmembrane receptors that connect cells with their surrounding environments. This superfamily of cell adhesion receptors recognizes and primarily binds ligands of ECM, including fibronectin, laminin, collagen, and vitronectin [18]. Integrins, as primary receptors for cellular adhesion to ECM molecules, act as crucial transducers for bidirectional cell signaling, regulating cell survival, differentiation, proliferation, migration, and tissue remodeling [19]. Generally, α and β subunits noncovalently bind to form an $\alpha\beta$ heterodimer, with two subunits involved in ligand recognition. Ligand binding head present in extracellular domain is connected by two arms, each linked to intracellular domain of integrin by a single transmembrane helix. All α and β subunits consist of numerous extracellular domains; α subunits of most integrins possess a domain of approximately 200 amino acids known as inserted (I) domain or von Willebrand factor A domain [20]. Cytoplasmic tails of human integrins typically contain fewer than 75 amino acids. However, the β4 tail contains more than 100 amino acids. Commonly, length of the extracellular portion is $\leq$788 amino acids for β subunits and 1,104 amino acids for α subunits [20]. In humans, integrins consist of 18 α and 8 β subunits that covalently attach to form 24 unique $\alpha\beta$

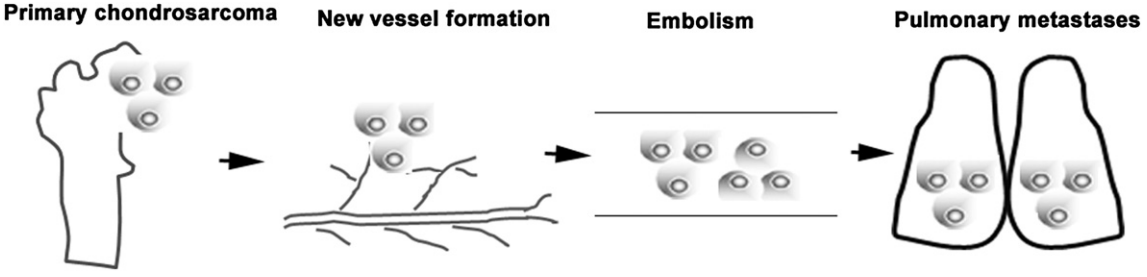

Fig. 1 – Metastasis of chondrosarcoma. Primary chondrosarcoma promotes new blood vessel formation. These blood vessels carry cancer cells to capillary beds in the lungs. Aggregates of tumor cells and other blood cells eventually form embolisms in distant lung capillaries. These cancer cells can then adhere to vascular endothelial cells to escape blood vessels. While entering the lung, these cells are exposed to factors in the microenvironment that support metastases.

transmembrane heterodimers, depending on cell type and cellular functions [21]. After ligand binding, integrin clustering occurs due to conformational changes, activating a signaling cascade and recruiting multiprotein complexes to focal adhesions.

Progression of several diseases is also related to modulation of integrin function and abnormal integrin expression: deleterious embryonic development, autoimmune diseases, cardiovascular diseases, and cancer [21]. Several studies reveal integrin signaling as altered in cancer cells to facilitate cancer progression by mediating metastasis, tumor invasiveness, tumor-induced angiogenesis, lymphangiogenesis, desmoplasia, and inflammation [22]. Integrins provide an appropriate tumor microenvironment during tumorigenesis via crosstalk with growth factor receptors and oncogenes. Modification in epigenetic regulation of integrin and integrin-linked kinase gene expression is directly related to carcinogenesis and cancer stem cell formation [22]. Activation and elevated expression of integrin-coupled signaling effectors have been implicated in a wide variety of human cancers, such as in the breast, colon, prostate, and ovaries [22]. Additionally, integrin has been implicated in metastasis of the lung, breast, bladder, and colon [23–25].

Integrins α2, α5, αv, β1, and β3, all highly expressed in chondrocytes, are the focus of multiple functional studies examining effect of integrin upregulation in chondrosarcoma cell migration [26–31]. Most data were acquired via Transwell cell migration and wound healing assays to examine migration and invasion activity of chondrosarcoma cells (JJ012 and SW1353 cell lines). Integrin αvβ3 expression is thought to be regulated by activation of transforming growth factor-β (TGF-β), stromal cell-derived factor-1 (SDF-1), leptin, glial cell-derived neurotropic factor, tumor necrosis factor-α (TNF-α), and interleukin-8 (IL-8) [26–31] (Table 1). Stimulation of cells with these factors induced cell migration and αvβ3 integrin expression in human chondrosarcoma cell lines. Pretreatment of cells with αvβ3 antibody or inhibitor (RGD) reduced cell motility of human chondrosarcoma cells; transfection of cells with αv or β3 integrin small interfering RNA (siRNA) also reduced cell migration of human chondrosarcoma cells. Yet αvβ3 integrin as a functional receptor induces expression of cysteine-rich protein 61 (Cyr61), connective tissue growth factor (CTGF) and osteopontin, along with nephroblastoma overexpressed (NOV)-mediated cell migration and invasion [32–35]. Hence, inside-out and outside-in integrin signaling is involved in metastasis of chondrosarcoma. Integrin α2β1 is a key factor in regulating migratory activity of chondrosarcoma cells. Prior results linked α2β1 integrin with cyclooxygenase-2 (COX-2)-, bradykinin-, and adiponectin-mediated cell migration of chondrosarcoma cells [36–38] (Table 1). Stimulation of cells with these factors increased cell surface and protein levels of α2β1 integrin in human chondrosarcoma cells. Cell migratory rates were reduced to control levels by addition of a monoclonal antibody against integrin α2β1. Furthermore, α5β1 integrin mediated insulin-like growth factor-I (IGF-I) cell motility of chondrosarcoma cells [39] (Table 1). Therefore, αvβ3, α2β1, and α5β1 are major integrin components in chondrosarcoma metastasis.

Table 1 – Integrin and metastasis of chondrosarcoma.

Integrin	Activators
αvβ3	TGF-β, SDF-1, leptin, GDNF, TNF-α, IL-8
α2β1	COX-2, bradykinin, adiponectin
α5β1	IGF-I

COX-2 = cyclooxygenase-2; IGF-I = insulin growth factor-I; IL-8 = interleukin-8; SDF-1 = stromal cell-derived factor-1; TGF = transforming growth factor-β; TNF = tumor necrosis factor-α.

2.2. MMPs and metastasis of chondrosarcoma

MMPs, or matrixins, a subfamily of metalloproteases, comprise 23 distinct proteases in humans. MMP was first identified in 1962 as responsible for degradation of fibrillar collagen in tadpole tails during metamorphosis and thus termed interstitial collagenase [40]. After identification of a similar collagenase in human skin, this protease was renamed MMP-1. MMPs have since been identified as major enzymes responsible for turnover of the ECM by proteolytic degradation of virtually all proteinaceous ECM components [41]. MMPs are primarily excreted proteins with several conserved domains. All contain a catalytic domain shielded by prodomain in an inactive form of the enzyme; this propeptide interacts with a catalytic region through conserved cysteine residue and Zn^{2+} ion in the catalytic pocket. Except for MMP-7, MMP-23, and MMP-26, all MMPs contain a C-terminal hemopexin-like domain that functions primarily as a substrate recognition sequence [42]. Although MMPs retain catalytic activity toward a wide range of substrates when this domain is absent, the hemopexin domain, which has a four-blade propeller structure with each blade consisting of four antiparallel β-sheets and one α-helix, is essential for degradation of triple-helical collagens [43]. Gelatinases (MMP-2 and -9) contain a series of three fibronectin type II inserts in the catalytic domain, which facilitate binding of gelatin and collagen [43].

MMP function is regulated at several levels. First, induction of gene expression is controlled by numerous growth factors and cytokines and may be suppressed by TGF-β and glucocorticoids. Recent studies indicate a pivotal modulatory role for epigenetic processes in MMP expression [44]. In addition to regulation by soluble factors, MMP expression may also be regulated by cell-cell contact or interaction of cells with ECM components such as ECM metalloprotease inducer or CD147 (EMMPRIN). Expressed MMPs are largely excreted as inactive proenzymes, with propeptide effectively limiting substrate entrance into and catalysis in the catalytic pocket by blocking the catalytic zinc (II) ion via a cysteine switch mechanism. Activation of proMMPs occurs through several mechanisms, all of which disrupt cysteine switch. The most important mechanism may be proteolytic removal of the prodomain by other endopeptidases such as furin [45]. Removal of the MMP prodomain, which contains a furin-like proprotein convertase recognition site, has been described for nine MMPs, including all membrane-type MMPs. Alternatively, the prodomain can be proteolytically removed by plasmin and other serine proteases or even other MMPs. This mechanism is well described for MMP-2 for which proMMP-2 binds the

endogenous MMP inhibitor tissue inhibitor of metalloproteases 2 (TIMP-2). This complex in turn serves as ligand for membrane-bound MMP-14 (or membrane type 1 MMP), leading to activation of MMP-2 [45].

MMPs play vital roles in many processes—cell proliferation, differentiation, apoptosis, migration—through degradation of both matrix and nonmatrix substrates [46–48]. These processing enzymes exhibit linkage to cancer and tumor progression, during which proteolysis of the ECM is required to accommodate increased growth, migration, and invasion of tumor cells. Expression of MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 has been demonstrated in human chondrosarcoma cells [49]. MMP-1 is particularly important in chondrosarcoma, as it is upregulated in locally invasive tumors and is responsible for degradation of collagen in cartilaginous tissue. Transfection of cells with MMP-1 siRNA reduced chondrosarcoma cell motility [50]. In contrast, thrombin and Wnt-induced secreted protein-1 (WISP-1) induced cell migration via upregulation of MMP-2 [51,52]. Stimulation of cells with thrombin and WISP-1 increased MMP-2 enzyme and protein expression. An MMP-2 siRNA and inhibitor also reduced motility of chondrosarcoma cells. MMP-3 is secreted as an inactive soluble proform, activated by a variety of proteases [49] (Table 2). Tang et al. [53] demonstrated that C-C motif ligand 5 (CCL5) enhances MMP-3 expression and cell migration. MMP-3 inhibitor or siRNA blocked CCL5-mediated cell migration and invasion. By contrast, MMP-9 upregulation is involved in osteopontin-mediated cell motility [34] (Table 2). Most effective for degrading type II collagen is MMP-13, thought to participate actively in situations that require rapid and effective remodeling of collagenous ECM is [49]. Previous studies show MMP-13 involved migration of human chondrosarcoma cells induced by bone morphogenetic protein-2 (BMP-2), Cyr61, CTGF, NOV, and IL-6 expression [32,33,35,54,55] (Table 2). MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 are major MMPs contributing to metastasis of chondrosarcoma cells.

2.3. *Intercellular adhesion molecule-1 and metastasis of chondrosarcoma*

ICAM-1 (also known as CD54), a member of the immunoglobulin (Ig) supergene family, is an inducible surface glycoprotein, primarily expressed in leukocytes, endothelial cells, and fibroblasts, which mediates adhesion-dependent, cell-cell interactions [56,57]. ICAM-1 possesses five extracellular Ig-like domains, a transmembrane segment, and a short cytoplasmic tail. Human ICAM-1 has five extracellular Ig-like domains, each presenting differences in ligand specificity: e.g., lymphocyte function-associated antigen-1 (LFA-1) antigen shows a binding preference for the first Ig domain, whereas Mac-1 binds to the third domain of extracellular regions of a molecule. The first Ig domain also harbors a binding site for major group human rhinoviruses and *Plasmodium falciparum*-infected erythrocytes [56,57]. The extracellular domain of ICAM-1 is crucial for transendothelial migration of leukocytes from the capillary bed into the tissue [58], and ICAM-1 may facilitate movement (or retention) of cells through the ECM [58].

The variable binding ability of ICAM-1 is directly related to the multifunctional physiologic and biologic roles of this molecule. Participation of ICAM-1 in inflammatory processes and in migration of activated leukocytes in inflammatory foci, first studied in the skin, is well established [59]. This complex process is mediated by several adhesive molecules expressed on both leukocyte and endothelial membrane. ICAM-1 is also a potent costimulatory molecule in T cell-mediated cytotoxicity; contribution of this adhesive molecule to host immune response led to a hypothesis that inhibition of ICAM-1 expression may correlate with cancer, because cancer is in fact a cascade of reactions closely connected to loss of normal immune surveillance. Conformational change in ICAM-1 and/or significantly altered concentration of its soluble form will positively correlate with breast cancer, hematologic malignancies, gastrointestinal cancer, and melanoma [59]. ICAM-1 plays a key role in lung cancer cell invasion [60]. ICAM-1 antibody or antisense ICAM-1 complementary DNA is likewise reported to reduce invasiveness of breast cancer cells [61]. ICAM-1 regulates motility of human chondrosarcoma cells [62]. Additionally, protein levels of ICAM-1 in human chondrosarcoma patients were significantly higher than in healthy patients. Treatment of chondrosarcoma with CCN6 demonstrably induces ICAM-1 mRNA and protein expression. Pretreatment of cells with ICAM-1 Ab or transfection of cells with ICAM-1 siRNA reduced CCN6-induced cell migration [62]; ICAM-1 thus plays an important role in motility of chondrosarcoma cells.

2.4. *COX-2 and metastasis of chondrosarcoma*

COXs are rate-limiting enzymes that catalyze conversion of arachidonic acid to prostaglandins (PGs). Two COX isoforms with distinct tissue distributions and physiologic functions have been identified [63]. COX-1 is constitutively expressed in many tissues and important for control of homeostasis [64]. Conversely, COX-2 is an inducible enzyme activated by extracellular stimuli such as growth factors and proinflammatory cytokines [65]. Recent investigations indicate overexpression of COX-2 frequently observed in many types of cancer (colon, lung, breast, pancreas, head, and neck) [66–68], typically associated with poor prognosis and short survival time. Four prostaglandin E (PGE) receptor subtypes have been identified (EP1-EP4) and their effects on human cancer cells analyzed [69]. Studies show EP1 as coupled to Ca^{2+} mobilization; EP2 and EP4 activate adenylate cyclase, whereas EP3 inhibits adenylate cyclase [69,70]. These studies indicate cancer cells expressing multiple subtypes of PGE receptor;

Table 2 – MMPs and metastasis of chondrosarcoma.

MMPs	Activators
MMP-2	Thrombin, WISP-1
MMP-3	CCL5
MMP-9	Osteopontin
MMP-13	BMP-2, Cyr61, CTGF, NOV, IL-6

BMP-2 = bone morphogenetic protein-2; CCL5 = C-C motif ligand 5; CTGF = connective tissue growth factor; Cyr61 = cysteine-rich protein 61; IL-6 = interleukin-6; MMP = matrix metalloproteinase; NOV = nephroblastoma overexpressed; WISP-1 = Wnt-induced secreted protein-1.

each subtype may link to diverse actions of PGE_2. Liu et al. [36] reported overexpression of COX-2 or exogenous PGE_2, increasing migration of human chondrosarcoma cells. Also, human chondrosarcoma tissues and chondrosarcoma cell lines significantly expressed COX-2 at levels above those in normal cartilage. Using pharmacologic inhibitors, activators, or genetic inhibition of EP receptors, Liu et al. found the EP1 receptor, not other PGE receptors, involved in PGE_2-mediated cell migration [36]. COX-2/PEG2/EP1 axis thus plays a vital role in metastasis of chondrosarcoma cells.

3. Discussion

Unlike in cases of mesenchymal malignancies such as osteosarcoma and Ewing sarcoma in which dramatic increases in long-term survival are reported with the advent of systemic chemotherapy, cases of chondrosarcoma continue to show poor prognosis because of lack of effective adjuvant therapy [12]. The metastatic potential of conventional chondrosarcomas correlates well with histologic grade of the tumor. Because of the relatively indolent growth rates of many low- and moderate-grade chondrosarcomas, approximately 15% of patients die of metastatic disease more than 5 years after initial diagnosis [12]. It thus is important to develop an effective adjuvant therapy for preventing chondrosarcoma metastasis. This article summarized recent studies examining metastasis of chondrosarcoma. Identifying signal pathways increases understanding of human chondrosarcoma metastasis, which may yield effective therapy.

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Review article

Long-term health outcomes of chronic hepatitis C patients: A review of findings from REVEAL-HCV cohort study

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ABSTRACT

Chronic hepatitis C affects more than 180 million people worldwide. As one of the most important infectious diseases, it causes around 250,000 deaths per year. A long-term follow-up cohort study is essential for evaluating health outcomes associated with virus infection, and for exploring potential seromarkers that have high predictability for risk of developing various diseases. However, the prospective cohorts consisted of individuals with chronic hepatitis C virus (HCV) infection are still rare. The Risk Elevation of Viral Load Elevation and Associated Liver Disease/Cancer in HCV (REVEAL-HCV) study has followed a cohort of 1095 residents seropositive for anti-HCV antibodies lived in seven townships in Taiwan for 15 years. These anti-HCV seropositives were asymptomatic and relatively more healthy than chronic hepatitis C patients cared in clinics and hospitals. Most of them acquired HCV infection through iatrogenic transmission routes in study townships. The epidemiological characteristics of HCV infection were very similar to those in countries with high prevalence such as Japan, Korea, Italy, and India. As the participants in the REVEAL-HCV study rarely received antiviral therapies, it provided an exceptional opportunity to study the natural history of chronic HCV infection. In this review article, we describe the details of participant enrollment, laboratory tests, follow-up procedures, and major recent findings. Anti-HCV seropositives with elevated serum HCV RNA levels were found to have an increasing risk of developing hepatocellular carcinoma in a dose-response relationship. In addition to the serum HCV RNA level, serum alanine aminotransferase levels and HCV genotype also had long-term predictability for the risk of hepatocellular carcinoma. Moreover, anti-HCV seropositives with detectable serum HCV RNA levels had an increased mortality from extrahepatic diseases such as cerebrovascular and renal diseases. Our study revealed that anti-HCV seropositives with detectable serum HCV RNA levels had an increased risk of hepatic and extrahepatic diseases.

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† Other members of the Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis C Virus (REVEAL-HCV) study are listed in the Appendix.

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1. Introduction

Hepatitis C virus (HCV) is recognized as a major cause of chronic liver disease. Liver cirrhosis eventuates in 20% to 30% patients with chronic HCV infection, generally after 2 to 3 decades [1]. Once cirrhosis occurs, hepatocellular carcinoma develops in 1% to 4% of these patients per year [2]. HCV was estimated to be attributable to one third of hepatocellular carcinoma cases globally [3]. Due to successful hepatitis B virus vaccination programs, HCV related health burdens are emerging quickly in Asian countries and represent a great public health burden [4]. Because a vaccine is not available and treatment options are still limited and expensive, the efforts of infection controls should be focused on primary prevention. A long-term follow-up cohort may help evaluate the incidence and mortality of various diseases associated with chronic HCV infection. In this review article, we describe the study population, enrollment and follow-up procedures, recent findings and future perspectives of Risk Elevation of Viral Load Elevation and Associated Liver Disease/Cancer in HCV (REVEAL-HCV) study.

2. REVEAL-HCV study cohort

The REVEAL-HCV study cohort was recruited from a community-based cancer screening program conducted in Taiwan during 1991 to 1992. There were seven townships selected as the study areas, including two northern townships (Sanchi and Chutung) and two southern townships (Potzu and Kaohsu) on main Taiwan Island, and three townships (Makung, Huhsi, and Paihsa) on Penghu Islets.

There were 89,293 inhabitants aged 30 to 65 years in the seven study townships invited to participate in the study, and 23,820 (11,973 males and 11,847 females) were enrolled after giving written informed consent. The vital status of the study participants were followed by the computerized linkage with the national cancer registration and death certification profiles. The national identification number, date of birth, and sex were used as the linking variables to double-check the vital status and causes of death of study participants. At enrollment, the participants were personally interviewed using structured questionnaires by well-trained public health nurses. The information collected included the sociodemographic characteristics (age, sex, educational levels, occupation, etc.), habits of life styles (cigarette smoking, alcohol consumption, betel nut chewing), and personal and family history of major diseases. Anthropometric measurements including weight and height were also performed.

In addition to the questionnaire interview, 10 mL blood samples were collected from each participant at study entry. The blood samples were obtained using disposable needles and heparinized vacuum syringes. They were fractioned on the day of collection and stored at −70°C until assayed. Serum samples of all participants were tested for hepatitis B surface antigen (HBsAg) by radioimmunoassay (Abbott Laboratories, North Chicago, IL, USA), anti-HCV by enzyme immunoassay (Abbott Laboratories), serum levels of aspirate aminotransferase (AST) and alanine aminotransferase (ALT) by serum chemistry autoanalyzer (Model 736, Hitachi, Tokyo, Japan) using commercial reagents (Biomerieux, Marcy L'Etoile, France).

Participants who were seropositive for anti-HCV were further examined for serum HCV RNA levels by polymerase chain reaction using the COBAS TaqMan HCV test, v2.0 (Roche Diagnostics, Indianapolis, NJ, USA), and an *in vitro* nucleic acid amplification test for the quantification of HCV RNA. The quantification method used the high pure system viral nucleic acid kit for manual specimen preparation and the COBAS TaqMan 48 Analyzer for automated amplification and detection. The manufacturer's procedures for sample preparation to extract HCV RNA, automated reverse transcription of the target RNA to generate complementary DNA, and amplification of target cDNA were followed. In any test procedure, a replicate of negative, low-positive, and high-positive controls were included in each run for HCV RNA quantification. The HCV RNA titer was expressed in international units (IU)/mL according to the WHO International Standard for HCV RNA NAT assays, and the linear range for the COBAS TaqMan HCV test was from 25 IU/mL to 3.9×10^8 IU/mL. Moreover, those with positive serum HCV RNA levels were examined for HCV genotypes by melting curve analysis, which could effectively differentiate different HCV genotypes by showing different melting temperatures [5,6]. In the REVEAL-HCV study, HCV genotype-1 and HCV genotype non-1 were differentiated.

Participants seropositive for HBsAg or anti-HCV were invited to receive regular health examinations. The health examinations included abdominal ultrasonography examinations and blood tests. The certified hepatologists performed the high-resolution real-time abdominal ultrasonography and interpreted according to a standardized protocol. Liver cirrhosis was determined based on a quantitative scoring system, which was derived from the appearance of liver surface (normal, irregular, undulated), liver parenchymal texture (normal, heterogeneous, coarse), intrahepatic blood vessel size (normal, obscure, narrowing), and splenic size (normal, enlarged) [7–9]. The serological tests included serum levels of AST, ALT, and α-fetoprotein. To ensure all study participants received standard care, those who had abnormal serum levels of ALT and/or α-fetoprotein levels and ultrasonographic findings were referred to hepatologists in medical centers for further clinical managements.

There were 1095 participants seropositive for anti-HCV but seronegative for HBsAg. Among them, 975 (89%) had adequate retrievable serum samples for HCV RNA test. Comparing those who had adequate serum samples ($n = 975$) and those without adequate serum samples for HCV RNA test ($n = 120$), there were no significant differences in the distributions of baseline characteristics except for gender. However, for the 975 anti-HCV seropositives with adequate samples for HCV RNA test, the proportion of gender was similar to that of all 1095 anti-HCV seropositives.

3. Seroprevalence of anti-HCV by age and gender

There were 1313 participants seropositive for anti-HCV, giving seroprevalence of 5.5% in our study population. The seroprevalence increased with age. For females, the seroprevalence of HCV was 3.0%, 3.6%. 4.2%, 6.8%, 7.3%, 9.7%, and 9.8%, respectively, for the 30 to 34, 35 to 39, 40 to 44, 45 to 49, 50 to 54, 55 to 59, and 60 to 65 year age groups. The corresponding seroprevalence for males was 2.7%, 3.7%, 3.2%, 5.2%, 5.6%, 6.4%, and 6.1%, respectively. As shown in Fig. 1, females had higher age-specific anti-HCV seroprevalence than males with the overall seroprevalence of 6.2% versus 4.8%, respectively.

The major risk factors of HCV infection in the REVEAL-HCV study population were iatrogenic risk factors including blood transfusion, hemodialysis, medical injections, and dental procedures. In our previous reports, >80% HCV infection could be attributable to iatrogenic factors [10,11]. Older people had an increased chance to receive multiple medical injections and had an increased cumulative risk of HCV infection in their lifetime. The gender difference in the seroprevalence of HCV infection might be explained by: 1) females being more concerned about their minor illness and more likely to receive glucose-based nutrient or vitamin injections than males, which were frequently prescribed to sick people; or 2) males infected with HCV having a higher mortality rate than infected females, thus the anti-HCV prevalence in males would more likely to be lower than females due to a faster attrition of the HCV-infected [12].

4. HCV RNA seropositive rate and its associated baseline characteristics

Serum HCV RNA was detectable in 676 (69.3%) anti-HCV seropositives in the REVEAL-HCV study cohort. Table 1 shows the HCV RNA seropositive rate by baseline characteristics. The HCV RNA seropositive rate was 78.8% in males and 62.0% in females, suggesting that females were more likely to have spontaneous seroclearance of HCV RNA. Participants with the habit of cigarette smoking or alcohol consumption had a higher HCV RNA seropositive rate than those without these habits. However, the associations might result from the higher proportions of cigarette smokers and alcohol drinkers in males than females. After adjustment for gender, there was no association between the HCV RNA seropositivity and habits of cigarette smoking and alcohol drinking. There was no significant association with HCV RNA seropositivity for body mass index (BMI) and history of diabetes.

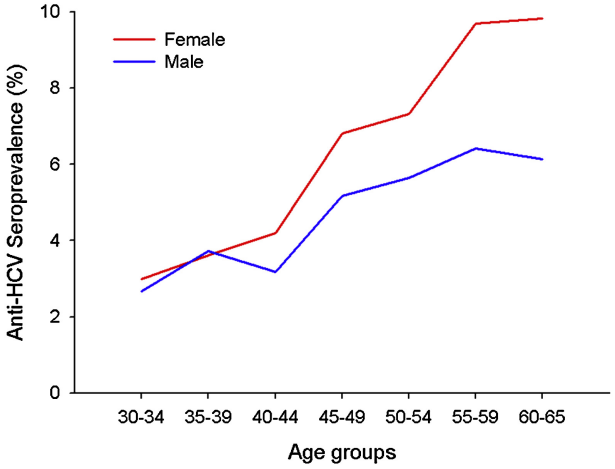

Fig. 1 – Seroprevalence of anti-HCV by age and gender.

Participants with increasing serum ALT levels had elevated HCV RNA seropositive rates. In comparison to those with serum ALT levels ≤15 U/L as the referent, the sex-adjusted odds ratio [95% confidence interval (CI)] of having detectable serum HCV RNA levels was 3.69 (2.69–5.06) and 10.18 (5.58–18.60), respectively, for serum ALT levels of 15 to 45 U/L and higher than 45 U/L. Males had a 2.26-fold (95% CI 1.66–3.07) higher risk of having detectable serum HCV RNA after adjustment for serum ALT levels. It is interesting to note that females had a higher anti-HCV seroprevalence, as shown in Fig. 1, but a lower HCV RNA seropositive rate among anti-HCV seropositives than males, as shown in Fig. 2. It suggests that the serum HCV RNA level might be a seromarker to be considered in management of anti-HCV seropositives. However, the importance and significance of this seromarker should be further evaluated by comparing the health outcomes between HCV RNA seronegative and seropositive participants who were seropositive for anti-HCV.

5. Incidence of hepatocellular carcinoma by baseline characteristics

There were 101 newly developed hepatocellular carcinoma (HCC) cases that occurred after 17,944 person-years of follow-up, giving an incidence rate of 562.9 per 100,000 person-years. Table 2 shows the number of participants, person-years of follow-up, number of HCC cases, and the incidence rate of HCC by baseline characteristics. Older individuals, or those with habits of cigarette smoking or alcohol consumption, increased BMI (≥25 kg/m^2), elevated serum ALT levels, or detectable serum HCV RNA levels had an increased incidence of HCC among the 1095 anti-HCV seropositives who were seronegative for HBsAg. The baseline characteristics that were significantly associated with increased HCC risk in univariate analyses were included in the subsequent multivariate analyses. Participants with older age, a habit of cigarette smoking or alcohol consumption, or increased BMI still had a significantly increased HCC risk after adjustment for each other, but no significant association was observed for habits of cigarette smoking or alcohol consumption after further adjustment for serum levels of ALT and HCV RNA.

In comparison to those with serum ALT levels ≤15 U/L as the referent group, the adjusted-hazard ratio (95% CI) was 1.78 (1.01–3.14) and 2.98 (1.65–5.40), respectively, for serum ALT levels of 16 to 44 U/L and ≥45 U/L. Individuals with detectable serum HCV RNA had 5.67 times greater risk of HCC than those with undetectable HCV RNA. In a recent study [13], it was found that serum levels of ALT and HCV RNA and HCV genotype had long-term predictability for HCC. These seromarkers had predictability 5 years or earlier than the

Table 1 – HCV RNA seropositive rates by baseline characteristics in REVEAL-HCV study.

Baseline characteristics	Total $n = 975$	HCV RNA undetectable $n = 299$ (30.7%)	HCV RNA detectable $n = 676$ (69.3%)	p value
Sex				
Females	550	209 (38.0%)	341 (62.0%)	<0.001
Males	425	90 (21.2%)	335 (78.8%)	
Age at recruitment, years				
30–39	163	55 (33.7%)	108 (66.3%)	0.49
40–49	217	72 (33.2%)	145 (66.8%)	
50–59	399	113 (28.3%)	286 (71.7%)	
60–65	196	59 (30.1%)	137 (69.9%)	
Cigarette smoking				
Never	701	245 (35.0%)	456 (65.0%)	<0.001
Yes	270	52 (19.3%)	218 (80.7%)	
Unknown	4	2	2	
Alcohol consumption				
No	893	286 (32.0%)	607 (68.0%)	0.003
Yes	80	13 (16.2%)	67 (83.8%)	
Unknown	2	0	2	
Body mass index (kg/m^2)				
<25	589	170 (28.9%)	419 (71.1%)	0.12
≥25	385	129 (33.5%)	256 (66.5%)	
Unknown	1	0	1	
History of diabetes				
No	930	285 (30.6%)	645 (69.4%)	0.78
Yes	42	12 (28.6%)	30 (71.4%)	
Unknown	3	2	1	
Serum ALT level (U/L)				
≤15	429	207 (48.3%)	222 (51.7%)	<0.001
16–45	387	79 (20.4%)	308 (79.6%)	
>45	159	13 (8.2%)	146 (91.8%)	

ALT = alanine aminotransferase.

occurrence of HCC. After 15 years of follow-up, the cumulative HCC risk was only 0.4% for participants seronegative for anti-HCV. There was an increasing cumulative HCC risk for anti-HCV-seropositive participants with undetectable, low, and high serum HCV RNA (1.1%, 6.4%, and 14.7%, respectively, $p < 0.001$ for trend). Among participants seropositive for anti-HCV, the cumulative HCC risk was 1.7%, 4.2%, and 13.8% for serum ALT levels of persistently ≤15 U/L, 15 to 45 U/L, and >45 U/L, respectively ($p < 0.001$ for trend). Among participants with detectable serum HCV RNA, the cumulative HCC incidence was 12.6% for HCV genotype 1 and 4.5% for genotype non-1 ($p < 0.001$) [13]. Moreover, the increasing HCC risk by the elevating serum levels of HCV RNA was found not only in men but also in women [14].

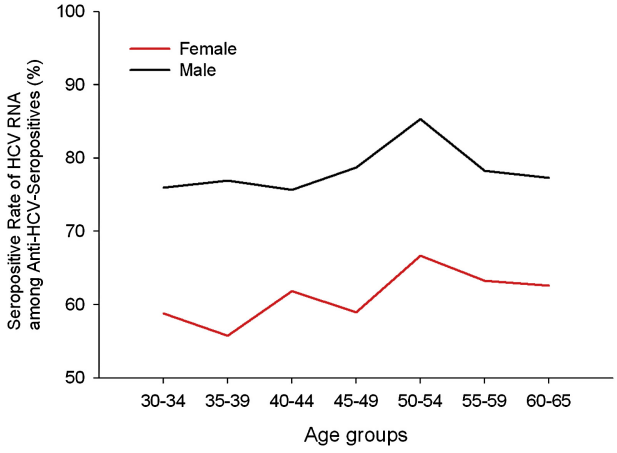

Fig. 2 – Seropositive rate of HCV RNA among anti-HCV-seropositives by age and gender.

6. All causes and liver-related mortality by baseline characteristics

Table 3 shows all-causes and liver-related mortality rates and associated hazard ratios for each baseline characteristic. The mortality rate from all causes was 1557.7 per 100,000 person-years and the liver-related mortality rate was 493.5 per 100,000 person-years, respectively. The cumulative mortality from all causes of death was 30.1% and 12.8% after 17 years of follow-up for participants with detectable and undetectable serum HCV RNA, respectively. Similarly, those with detectable serum HCV RNA had an increased cumulative mortality from liver-related diseases compared with those with undetectable

Table 2 – Numbers of participants, person-years of follow-up, hepatocellular carcinoma case numbers and incidence rates by baseline characteristics.

Baseline risk factors	No. (%) of participants	Pearson-years of follow-up	No. of hepatocellular carcinoma cases	Incidence rate per 100,000 person-years	Crude hazard ratio (95% CI)	Multivariate adjusted hazard ratio (95% CI)
Sex						
Females	630 (57.5)	10430	51	489.0	1.00	Not included
Males	465 (42.5)	7514	50	665.4	1.36 (0.92–2.01)	
Age at recruitment, years						
30–39	186 (17.0)	3148	3	95.3	1.00	1.00
40–49	243 (22.2)	4055	11	271.3	2.86 (0.80–10.26)	2.45 (0.68–8.80)
50–59	444 (40.6)	7196	56	778.2	8.32 (2.60–26.57)	5.70 (1.77–18.37)
60–65	222 (20.3)	3546	31	874.3	9.36 (2.86–30.62)	6.78 (2.05–22.39)
Cigarette smoking						
Never	793 (72.7)	13092	65	496.5	1.00	1.00
Ever	298 (27.3)	4782	36	752.8	1.52 (1.01–2.29)	1.12 (0.70–1.80)
Alcohol consumption						
No	1007 (92.1)	16540	87	526.0	1.00	1.00
Yes	86 (7.9)	1371	13	948.3	1.83 (1.02–3.28)	1.38 (0.69–2.76)
Body mass index (kg/m^2)						
<25	663 (60.7)	11003	45	409.0	1.00	1.00
≥25	430 (39.3)	6906	56	810.9	2.01 (1.36–2.97)	1.76 (1.16–2.67)
History of diabetes						
No	1043 (95.7)	17115	95	555.1	1.00	Not included
Yes	47 (4.3)	747	6	802.7	1.48 (0.65–3.37)	
Serum ALT level (U/L)						
≤15	495 (45.2)	8302	21	252.9	1.00	1.00
16–44	426 (38.9)	6950	43	618.7	2.49 (1.48–4.19)	1.78 (1.01–3.14)
≥45	174 (15.9)	2692	37	1374.4	5.66 (3.31–9.67)	2.98 (1.65–5.40)
Serum HCV RNA level (IU/mL)						
<25 (undetectable)	299 (30.7)	5040	5	99.2	1.00	1.00
≥25 (detectable)	676 (69.3)	10943	86	785.9	8.08 (3.28–19.90)	5.67 (2.25–14.31)

ALT = alanine aminotransferase.

serum HCV RNA, with the cumulative mortality of 12.8% and 1.6% [15]. Generally, male gender, older age, habits of cigarette smoking and alcohol consumption, BMI ≥25 kg/m^2, history of diabetes, elevated serum ALT levels, and detectable serum HCV RNA levels were associated with an increased mortality from all causes or liver-related deaths. After adjustment for potential risk factors, participants with detectable serum HCV RNA had an increased risk for all causes of death and hepatic-related deaths with the adjusted hazard ratio (95% CI) of 2.78 (1.56–3.33) and 6.53 (2.32–18.37), respectively. This implies that those with detectable serum HCV RNA levels might die from other extrahepatic diseases in addition to hepatic diseases. Our findings indicate that the serum HCV RNA level is an important marker for management of individuals seropositive for anti-HCV.

7. HCV infection and deaths from extrahepatic diseases

The mortality from extrahepatic diseases was 1064.2 per 100,000 person-years for the participants in the REVEAL-HCV cohort. Among participants seronegative for HBsAg, the cumulative mortality from extrahepatic diseases after 17 years of follow-up was 12.2% for anti-HCV seronegatives and 17.7% for anti-HCV seropositives. In other words, participants seropositive for anti-HCV had a 1.4-fold increased risk of dying from extrahepatic diseases after adjustment for age and sex. Anti-HCV seropositives had an increased mortality from either extrahepatic cancers or extrahepatic diseases other than cancers with age-sex-adjusted hazard ratio (95% CI) of 1.23 (0.92–1.65) and 1.43 (1.19–1.73), respectively. Fig. 3 shows the associations between extrahepatic deaths and HCV infection. The HCV infection was associated with an increased mortality from circulatory diseases. Based on the long-term follow-up design of the REVEAL-HCV study, there was a correct causal temporality for the HCV-induced atherosclerotic diseases [16]. Moreover, the increasing serum HCV RNA levels were found to be associated with mortality from cerebrovascular disease in a dose-response relationship after adjustment for conventional risk factors for cerebrovascular disease. Compared with participants seronegative for anti-HCV as the referent group, the multivariate-adjusted hazard ratio (95% CI) of dying from cerebrovascular disease was 1.43 (0.63–3.23), 2.29 (1.38–3.82), and 2.81 (1.25–6.35), respectively, for anti-HCV-seropositive participants with undetectable, low, and high serum levels of HCV RNA ($p < 0.001$ for trend). However, there was no significant association between HCV genotype and mortality from cerebrovascular disease [17]. In addition, HCV infection was associated with an increased mortality from renal disease and cancers of the esophagus, prostate, and thyroid, and the mortality was even higher for those with detectable serum of HCV RNA [15].

Table 3 – All-causes and liver-related mortality in patients with HCV infection by baseline characteristics.

Baseline risk factors	All causes of death				All liver-related deaths			
	No. of deaths	Mortality rate per 100,000 person-years	Crude HR (95% CI)	Multivariate adjusted HR (95% CI)	No. of deaths	Mortality rate per 100,000 person-years	Crude HR (95% CI)	Multivariate adjusted HR (95% CI)
Sex								
Females	118	1187.0	1.00	1.00	39	392.3	1.00	1.00
Males	144	2093.3	1.80 (1.41–2.30)	1.30 (0.90–1.88)	44	639.6	1.69 (1.10–2.60)	1.04 (0.54–1.99)
Age at recruitment, years								
30–39	18	592.1	1.00	1.00	3	98.7	1.00	1.00
40–49	28	712.9	1.21 (0.67–2.19)	1.19 (0.62–2.27)	5	127.3	1.30 (0.31–5.43)	1.10 (0.26–4.63)
50–59	129	1940.3	3.38 (2.06–5.53)	2.96 (1.72–5.10)	49	737.0	7.80 (2.43–25.01)	5.20 (1.20–16.88)
60–65	87	2715.6	4.85 (2.92–8.06)	4.29 (2.45–7.51)	26	811.5	8.96 (2.71–29.61)	6.74 (2.01–22.61)
Cigarette smoking								
Never	156	1250.9	1.00	1.00	51	409.0	1.00	1.00
Ever	106	2477.4	2.05 (1.60–2.62)	1.35 (0.92–1.98)	32	747.9	1.93 (1.24–3.00)	1.30 (0.65–2.62)
Alcohol consumption								
No	228	1460.3	1.00	1.00	71	454.8	1.00	1.00
Yes	34	2900.7	2.07 (1.44–2.96)	1.71 (1.14–2.57)	12	1023.8	2.38 (1.29–4.39)	1.77 (0.85–3.67)
Body mass index (kg/m^2)								
<25	140	1355.3	1.00	1.00	38	367.9	1.00	1.00
≥25	121	1873.6	1.40 (1.10–1.79)	1.35 (1.04–1.76)	45	696.8	1.94 (1.26–2.99)	1.84 (1.16–2.93)
History of diabetes								
No	230	1422.5	1.00	1.00	77	476.2	1.00	1.00
Yes	31	5404.8	4.26 (2.93–6.21)	3.99 (2.62–6.08)	6	1046.1	2.62 (1.14–6.03)	2.27 (0.90–5.70)
Serum ALT level (U/L)								
≤15	91	1163.8	1.00	1.00	19	243.0	1.00	1.00
16–44	108	1662.5	1.46 (1.10–1.92)	1.03 (0.76–1.41)	32	492.6	2.08 (1.18–3.68)	1.37 (0.75–2.53)
≥45	63	2515.3	2.24 (1.63–3.09)	1.24 (0.86–1.78)	32	1277.6	5.54 (3.14–9.78)	2.72 (1.46–5.05)
Level of HCV RNA (IU/mL)								
<25 (undetectable)	36	744.5	1.00	1.00	4	82.7	1.00	1.00
>25 (detectable)	194	1902.2	2.63 (1.84–3.75)	2.78 (1.56–3.33)	70	686.4	8.67 (3.16–23.73)	6.53 (2.32–18.37)

ALT = alanine aminotransferase.

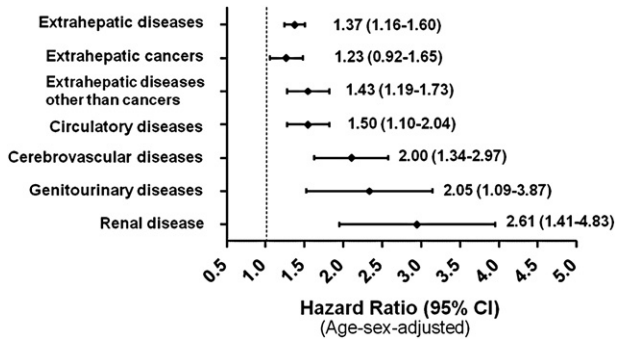

Fig. 3 – Hepatitis C virus infection and mortality from extrahepatic diseases.

8. Advantages and limitations of the REVEAL-HCV study

Chronic hepatitis C patients in Taiwan rarely received antiviral treatment with interferon due to its high cost and adverse effects until November 2003, when patients with abnormal serum ALT levels (>82 U/L) and moderate fibrosis proven by liver biopsy could be reimbursed for treatment by the National Health Insurance. Therefore this cohort study may be considered as a natural history study of chronic hepatitis C. To ensure that study participants received standard care, those who had abnormal serum levels of ALT and α-fetoprotein or abnormal ultrasonographic findings were referred to medical centers for further clinical management in this study. This cohort, consisting of 1000 anti-HCV seropositives, provided an exceptional opportunity to examine the seromarker changes and liver disease occurrence of anti-HCV seropositives during the natural course of HCV infection.

Participants enrolled in the REVEAL-HCV cohort lived in the community. Unlike other cohorts, which enrolled patients with experiences of drug injections [18] or HCV-contaminated vaccinations [19,20], the exact time of HCV infection was unavailable for our participants. As the major risk factors of HCV infection in the REVEAL-HCV cohort were iatrogenic factors, it was difficult to obtain the exact time of HCV infection; the information on advanced fibrosis or mild cirrhosis was not available in this community-based cohort because it is not practical to have asymptomatic participants examined by liver biopsy. Liver cirrhosis is an intermediate clinical outcome before the occurrence of HCC among chronic hepatitis C patients. Based on the abdominal ultrasonographic examination and serial tests of serum levels of AST and ALT, >80% of newly-developed HCC cases in participants seropositive for anti-HCV had liver cirrhosis detected by ultrasonography and/or an increased ratio between serum levels of AST and ALT.

9. Summary

Based on the REVEAL-HCV cohort study, we found that anti-HCV seropositives with detectable serum HCV RNA levels had an increased risk of both hepatic and extrahepatic diseases. Anti-HCV seropositives with undetectable serum HCV RNA levels had cumulative HCC risk similar to anti-HCV seronegatives (1.1 vs. 0.4%), implying that antiviral treatment to aid seroclearance of HCV RNA may benefit patients. In addition, the findings suggest that clinical patients experienced sustained virologic response after receiving antiviral therapy may have reduced HCC risk and improved survival [21–26]. Recent trials showed that use of direct-acting antiviral agents may achieve sustained virologic response among patients who had not had a response to prior therapy [27,28]. Our study provides evidence that patients with HCV infection, particular for those with active HCV infection (seropositive for HCV RNA), should be encouraged for intensive management because they had an increased risk of HCC and mortality from hepatic or extrahepatic diseases. We also found that the prevalence of anti-HCV in a community was associated with HCV RNA seropositive rate among anti-HCV seropositives in the community, suggesting that anti-HCV seropositives with detectable serum HCV RNA levels played a major role in the transmission of the virus in the community [12]. For the control of hepatic or extraheptic diseases and virus infection, anti-HCV seropositives should be tested for serum HCV RNA levels by a sensitive assay. Those with active HCV infection should be instructed to be aware of HCV-related health outcomes and HCV transmission routes as well as the need to take actions for HCV RNA seroclearance.

10. Future perspectives

Recently, human genetic variants predicting successful treatments have been identified by genome-wide association study (GWAS) from several independent study groups [29–31]. They studied different ethnic populations and found that genetic variants near the IL28B gene were associated with antiviral response in patients infected with HCV genotype 1. Two single nucleotide polymorphisms (SNPs) near the interleukin 28B gene (IL28B, also called IFNλ3), rs12979860 and rs8099917, were associated with antiviral treatment response in chronic hepatitis C patients [29–31]. The C allele of the SNP (rs12979860) was found to be associated with the spontaneous clearance of HCV in a follow-up study [32]. A recent study showed that Taiwanese patients with chronic hepatitis C receiving antiviral therapy have a lower daily viral production rate than western patients, and the rs8099917 TT genotype may contribute to the increased viral clearance rate and better virological responses [33]. These findings imply that host genetic factors may be involved in the natural course of HCV infection and the pathogenesis of liver diseases. IL28B polymorphism (T allele) seems to be involved in the development of HCV-induced HCC and the course of HCV recurrence after liver transplantation in a recent study [34]. In Taiwan, most chronic hepatitis C patients carried the favorable genotype associated with better treatment responses and the minor allele frequency (T of rs12979860 and G of rs8099917) were very rare [35–38]. To better understand the associations between the SNPs near IL28B and the risk of liver cirrhosis or HCC, a study with a large sample size is needed. In addition to the IL28B gene, a recent GWAS conducted in Japan identified SNPs associated with the occurrence of hepatocellular carcinoma among chronic hepatitis C patients [39,40]. It will be

interesting to discover these genetic variants to understand the pathogenesis of liver disease progression further or to apply them as diagnostic or risk predictive biomarkers [41]. Although high-throughput technologies to discover human genetic variants have developed rapidly to accelerate the genotyping, validation of genetic markers in other external populations is still essential and functional studies are needed. Moreover, to stratify high-risk patients who need intensive care is essential. Recently, several study groups focus on the development of prediction models for liver-related outcomes among chronic hepatitis C patients [41–47], which may aid physicians to communicate with patients and enhance patients' compliance to receive standard care. In Taiwan, pegylated-interferon plus ribavirin is the standard care for chronic hepatitis C patients [48,49]. The sustained virologic response rate for patients with genotype 1 was around 70% [25,49]. It will be important to follow the subsequent risk for liver-related outcomes among patients with sustained virologic response or with nonvirologic response as well as to compare the disease burdens occurred in patients with treatment experiences or not [50]. Collaborative studies to understand the diseases associated with HCV infection better and to promote appropriate clinical managements of chronic hepatitis C patients are in urgent need.

Appendix

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Review article

Anticancer potential of emodin

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ABSTRACT

Traditional Chinese Medicine (TCM) is widely used in clinical research due to its low toxicity, low number of side effects, and low cost. Many components of common fruits and vegetables play well-documented roles as chemopreventive or chemotherapeutic agents that suppress tumorigenesis. Anthraquinones are commonly extracted from the Polygonaceae family of plants, e.g., *Rheum palmatum* and *Rheum officinale*. Some of the major chemical components of anthraquinone and its derivatives, such as aloe-emodin, danthron, emodin, chrysophanol, physcion, and rhein, have demonstrated potential anticancer properties. This review evaluates the pharmacological effects of emodin, a major component of *Aloe vera*. In particular, emodin demonstrates anti-neoplastic, anti-inflammatory, anti-angiogenesis, and toxicological potential for use in pharmacology, both *in vitro* and *in vivo*. Emodin demonstrates cytotoxic effects (e.g., cell death) through the arrest of the cell cycle and the induction of apoptosis in cancer cells. The overall molecular mechanisms of emodin include cell cycle arrest, apoptosis, and the promotion of the expression of hypoxia-inducible factor 1α, glutathione S-transferase P, N-acetyltransferase, and glutathione phase I and II detoxification enzymes while inhibiting angiogenesis, invasion, migration, chemical-induced carcinogen-DNA adduct formation, HER2/neu, CKII kinase, and p34cdc2 kinase in human cancer cells. Hopefully, this summary will provide information regarding the actions of emodin in cancer cells and broaden the application potential of chemotherapy to additional cancer patients in the future.

1. Introduction

Numerous researchers have reported the use of phytochemical compounds such as anthraquinone emodin extracts from traditional Chinese medicines (TCM), including *Polygonum multiflorum* [1,2], *P Cuspidatum* [3,4], *Rumex patientia* [5], *Rhamnus catharticus*, *Rhamnus orbiculatus* [6], *Aloe vera* [7], *Acorus tatarinowii* [8], *Cassia obtusifolia* [9], *Cassia occidentalis* [10], *Rheum palmatum* [11], *Rheum officinale* [12], *Eriocaulon buergerianum* [13], *Dendrobium thyrsiflorum* [14], *Fibraurea tinctoria* [15],*Coptis chinensis* [16],*Scutellaria baicalensis* [16], *Isatis indigotica* [17], and *Rumex chalepensis* [18]. Studies on the use of TCM have noted lipid regulation activities and anti-inflammatory, antimicrobial, antiviral, antitumor, and antioxidant effects. To learn more about the therapeutic functions of TCM, experiments are needed to identify the functional ingredients and ascertain the

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molecular mechanisms of these compounds. Recent research is paying more attention to TCM because it may have future applications in clinical medicine. In particular, rhubarb (*Rheum palmatum*) is one of the oldest and most famous Chinese herbal medicines and is still used in various herbal remedies and therapeutic applications. Based on current reports and investigation, we believe rhubarb has clinical potential.

Rhubarb is a well-known treatment for many diseases in TCM [19,20]. Anthraquinones extracted from the rhubarb rhizome exhibit antidiabetic properties, suggesting a metabolic role in the insulin-stimulated glucose transport pathway [21]. Both *in vitro* and *in vivo* studies have reported the antimicrobial activities of extracts from *Sapindus mukorossi* and *Rheum emodin* against *Helicobacter pylori* [22]. Moreover, the antioxidant and anticancer potential of *Rheum emodin* rhizome extracts have demonstrated therapeutic value [23]. Extracts from *Rheum palmatum* have a high level of inhibitory activity against anti-Severe acute respiratory syndrome (SARS) coronavirus 3C-like protease effects [24]. A polysaccharide extracted from *Rheum tanguticum* has been shown to affect 2,4,6-trinitrophene sulphonic acid (TNBS)-induced colitis and $CD4^+$ T cells in rats [25]. Rhubarb has also demonstrated protective effects against experimental severe acute pancreatitis [26]. A study on anti-Oketsu activity indicates that rhubarb II has inhibitory effects against allergies [27]. Hexane extracts from *Rheum undulatum* not only decreases cell viability, thereby triggering apoptotic cell death in oral cancer, but also decreases the expression of specificity protein (Sp1) and its downstream protein, survivin [28].

The effects of rhubarb extracts on experimental chronic renal failure (CRF) indicate that it can reduce proteinuria and the severity glomerulosclerosis within remnant kidneys in rats [29]. Treatment of menopausal symptoms using an extract from the roots of *Rhapontic rhubarb* (plus the results of *in vitro* and *in vivo* experiments) indicate estrogenic actions, especially estrogen receptor β (ERβ)-mediated effects [30]. Oligostilbenes from rhubarb also inhibit low-density lipoprotein and high-density lipoprotein oxidation humans [31], suggesting a pivotal role in the prevention of lipoprotein oxidation.

2. Active ingredients found in the Polygonaceae family

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) (Fig. 1) is an active ingredient in the root and rhizome of *Rheum palmatum* (Polygonaceae) [11]. This herb has been used in TCM for the treatment of gallstones, inflammation, hepatitis, and osteomyelitis and is also a known vasorelaxant and diuretic [32]. It reportedly has antibacterial, anti-inflammatory, antiviral, anti-ulcerogenic, anticancer, immunosuppressive [33–36], and chemopreventive effects [37]. Emodin has also been reported to exert inhibitory effects on cell death in the human lung squamous carcinoma CH27 cell line [36], and-human promyeloleukemic HL-60 cells induce apoptosis by activating the caspase-3 cascade independently of reactive oxygen species (ROS) production [38]. Emodin-induced apoptosis in human cervical cancer Bu 25TK cells occurs through poly (ADP-ribose) polymerase cleavage and the activation of caspase-9, but caspase-8 is not activated [39].

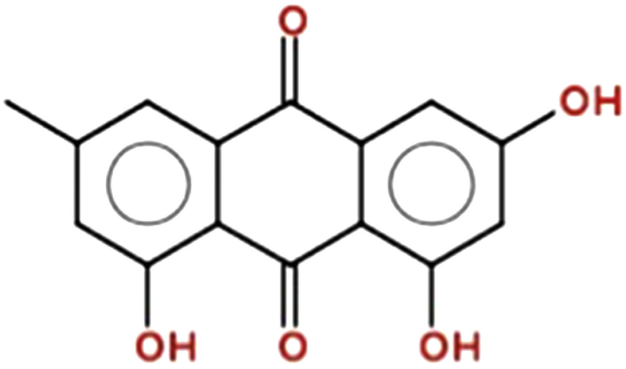

Fig. 1 – Chemical structure of emodin.

Moreover, emodin triggers apoptosis in human hepatoma HepG2/C3A, PLC/PRF/5, and SK-HEP-1 cells through a p53-dependent pathway [40]. In addition, emodin enhances arsenic trioxide-induced apoptosis by generating ROS and inhibiting survival signaling [41], and gene expression alteration occurs in HeLa cells through the redox-dependent enhancement of arsenic cytotoxicity [42]. Our laboratory has proven that Aloe-emodin affects the expression of cytokines and the functions of leukocytes in Sprague Dawley rats [134]. Emodin affects murine myelomonocytic leukemia WEHI-3 cells *in vitro* and enhances phagocytosis in leukemic mice *in vivo* [135].

Emodin downregulates androgen receptors and inhibits the cellular growth of prostate cancer [43]. Emodin inhibits the adhesion of human breast cancer (MDA-MB-231), human cervix epithelioid carcinoma (HeLa), and human hepatocarcinoma (HepG2) tumor cells by suppressing lipid raft coalescence and interfering with integrin clustering and focal adhesion complex (FAC) formation [44]. Likewise, it has been demonstrated that emodin could act as a Janus-activated kinase 2 inhibitor and have cytotoxic activities against multiple myeloma in humans [45]. Emodin selectively inhibits the interleukin–6-induced JAK2/STAT3 pathway and induces apoptosis in myeloma cells via the downregulation of myeloid cell leukemia 1 (Mcl-1) cells [45]. In local ischemic myocardium, emodin mediates protection from acute myocardial infarction through the inhibition of inflammation and apoptosis [46].

3. Pharmacological mechanisms against various types of cancer cells

Emodin has shown significant anticancer activities in several tumor cells, both *in vivo* and *in vitro*, while its molecular anticancer mechanisms have not been well explored. This review discusses emodin's pharmacological activities and the mechanisms that induce cell death in many types of human cancer cells, both *in vitro* and *in vivo*. Research findings on emodin-induced cytotoxicity and its protective effects are described below.

3.1. *HER2/neu expression*

Previously published reports in the literature confirm that emodin and its derivatives inhibit p185neu tyrosine kinase via

the suppression of HER2/neu-transformed phenotypes (e.g., by inducing cellular transformations and metastasis-associated potential) [47]. In breast cancer, the emodin derivative, azide methyl anthraquinone, induces mitochondrion-dependent apoptosis in HER2/neu-overexpressing MDA-MB-453 cells and lung adenocarcinoma Calu-3 cells and blocks HER2/neu binding to Hsp90. Azide methyl anthraquinone also induces the proteasomal degradation of HER2/neu in MDA-MB-453 and Calu-3 cells *in vitro* [48].

3.2. *CKII and p34cdc2 kinase*

Emodin inhibits the activity of casein kinase II (CKII) by acting as a competitor at ATP-binding sites. [49]. CKII is involved in the proliferation of human U87 astroglioma cells via stimulation of basal phospholipase D (PLD) activity. [50]. Emodin reportedly induces apoptosis in human tongue squamous cancer SCC-4 cells through ROS and mitochondria-dependent pathways *in vitro* [51]. Aloe-emodin, which is extracted from the rhizome of *Rheum palmatum*, downregulates MMP-2 through a p38 Mitogen-activated protein kinase (MAPK)-Nuclear factor-κB (NF-kB)–dependent pathway, thereby leading to the inhibition of invasion by nasopharyngeal carcinoma cells (NPC-TW 039 and NPC-TW 076) [52].

3.3. *Oncogenes*

It is well documented that nuclear factor-kappaB (NF-κB) plays an important role in the transcription of tumor cells [53,54]. It has been reported that emodin inhibits the proliferation and induction of apoptosis in pancreatic cancer cell lines (SW1990/GZ and SW1990). Emodin not only downregulates NF-κB under unstimulated conditions, but it also inhibits gemcitabine-induced NF-κB protein expression [53]. Aloe-emodin also purportedly induces antiproliferative activities through p53- and p21-dependent apoptotic pathway in the human hepatoma HepG2 and Hep3B cell lines [55]. An attractive target of oncogene-based anticancer drugs derived from natural herbal plants (like emodin), *Polygonum cuspidatum* exhibits strongly selective activities against src-HER2/neu and ras-oncogenes. In other words, emodin might be a oncogenetic signal for the inhibition of transduction [56].

3.4. *Hypoxia-inducible factor 1α*

Heterodimer hypoxia-inducible factor 1α (HIF-1) consists of a β subunit that is constitutively expressed and an oxygen-regulated α subunit. HIF-1 regulates genes that participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival [57]. The activity of HIF-1, especially its α subunit, is controlled by the posttranslational modification of the amino acid residues in its subunits [57]. HIF-1 plays a key role in the cellular response to tumor hypoxia that poses a major problem to successful radiotherapy and chemotherapy. The targeting of HIF-1 is now considered to be a pivotal and efficient strategy for treating neurodegenerative maladies like Alzheimer's (AD), Parkinson's (PD), Huntington's Disease (HD), amyotrophic lateral sclerosis (ALS), etc. [58]. It has also been reported that emodin diminishes hypoxia-induced embryotoxicity by upregulating HIF-1 and intracellular superoxide dismutases in whole cultured mouse embryos [59]. As a novel inhibitor of HIF-1, emodin is an adjunct that boosts the efficacy of cytotoxic drugs used for the treatment of prostate cancer DU-145 cells, demonstrating overactivated HIF-1 and potent multidrug resistance (MDR) [60].

3.5. *N-acetyltransferase activity*

Our previous studies have demonstrated how emodin and aloe-emodin inhibit N-acetyltransferase (NAT) activity and gene expression in mouse leukemia L1210 cells [61], human melanoma cells (A375.S2) [62], and strains of *H pylori* in peptic ulcer patients [63,64].

3.6. *Cell cycle arrest*

The cell cycle is classified into the G0/G1, S, and G2/M phases; if an agent induces apoptosis, then those will be sug-G1 phase [65]. In clinic settings, some anticancer agents can induce cell cycle arrest (arrest during the G0/G1, S, and/or G2/M phase) [65,66]. It has been reported that emodin and docosahexaenoic acid (DHA) increase arsenic trioxide interferon–α-induced cell death in human T-cell leukemia virus type 1 (HTLV-I)–transformed cells via ROS generation and the inhibition of Akt and activator protein 1 (AP-1) [67]. Emodin inhibits the growth of hepatocellular carcinomas, such as Huh7, Hep3B, and HepG2, through anticancer pathways (e.g., G2/M arrest and increased expression levels of the involved genes, both at the mRNA and protein levels) [68]. Emodin also reportedly inhibits vascular endothelial growth factor-A–induced angiogenesis [69]. Other investigators have demonstrated how emodin induces apoptosis through the p53-dependent pathway in human hepatocellular carcinoma cells [40], as well as growth arrest and death through ROS and p53 in human vascular smooth muscle cells [70].

Aloe-emodin also induces G2/M arrest in human promyelocytic leukemia HL-60 cells [71], cervical cancer HeLa cells [72], and through activated alkaline phosphatase in human oral cancer KB cells *in vitro* [73]. It has also been reported that aloe-emodin induces apoptosis through protein 53 (p53)-dependent apoptotic pathways in human bladder cancer T24 cells [74]. Aloe-emodin induces destabilization of caspase-8 and -10-associated RING protein (CARP) mRNA, indicating that caspase-8–mediated p53-independent apoptosis in human carcinoma cells [75] and human nasopharyngeal carcinoma cells induces caspase-3, -8, and -9–mediated activation of the mitochondrial death pathway [76]. Still, the antiproliferative activity of aloe-emodin occurs via p53- and p21-dependent apoptotic pathways in human hepatoma HepG2 cell lines [55,77]. Other evidence indicates that aloe-emodin and emodin inhibit schisandrin B in gastric cancer cells *in vitro* [78].

3.7. *Apoptosis*

It is well documented that the best strategy for killing cancer cells is via the induction of apoptosis [79] and that the best way for chemotherapeutic agents to kill cancer cells is to trigger apoptosis in tumors [79,80]. In human hepatoma Huh-7 cells, apoptosis is mediated by the downregulation of calpain-2 and ubiquitin-protein ligase E3A [81]. Emodin has strong antioxidative and anticancer actions and abrogates cisplatin-

induced nephrotoxicity in rats [82]. Other reports have cited the antitumor and apoptosis-promoting properties of emodin, an anthraquinone derivative, against pancreatic cancer in mice by inhibiting Akt activation [12]. Emodin enhances apoptosis in cisplatin-induced gallbladder carcinomas in a ROS-dependent manner and suppresses survivin expression [83]. Emodin downregulates X-linked inhibitor of apoptosis protein (XIAP) expression [84] and inhibits NF-κB against human pancreatic cancer [53], thereby enhancing the antitumor efficacy. Emodin induces apoptosis in the mouse microglial BV-2 cell line via Tribbles homolog 3 (TRB3) and eliminates inflammatory microglia, thereby exerting neuroprotective effects [85].

Emodin induces ROS generation and the activation of the ATM-p53-Bax–dependent signaling pathway in human lung adenocarcinoma A549 cells [86]. It has been reported that emodin exerts potential anticancer effects in pancreatic cancer cells by downregulating the expression of survivin and β-catenin [87]. Emodin also demonstrates potential as an antiatherosclerosis agent by inhibiting the proliferation of Tumor necrosis factor (TNF)-alpha–induced human aortic smooth muscle cells (HASMC) through mitochondrial- and caspase-dependent apoptotic pathways [88]. Emodin induces apoptosis via the caspase-3–dependent pathway in human renal proximal tubule HK-2 cells [89] and inhibits human prostate cancer LNCaP cell proliferation via androgen receptor and p53-p21 pathways [90]; pyrazole emodin derivatives inhibit the growth of and induce apoptosis in human hepatocellular carcinoma HepG2 cells [91]. Pyrazole emodin derivative also induce apoptosis in human cervical cancer cells via the activation of caspase-3 and -9 and the cleavage of poly (ADP-ribose) polymerase [39]. Aloe-emodin induces apoptosis in human lung nonsmall carcinoma H460 cells through Cyclic Adenosine monophosphate (cAMP)-dependent protein kinase, protein kinase C, Bcl-2, caspase-3, and the p38 signaling pathway and induces human lung squamous cell carcinoma CH27 cell death via the Bax and Fas death pathways [92,93]. Emodin not only successfully suppresses acute graft rejection *in vivo*, thereby prolonging the survival of the recipient rats by inhibiting hepatocellular apoptosis and modulating Th_1/Th_2 balance [94], but also mediates protection against acute myocardial infarction [46] in local ischemic myocardium. Emodin can reverse gemcitabine resistance in pancreatic cancer cells via mitochondria-dependent pathways *in vitro* [95].

3.8. *Glutathione S-transferase and glutathione peroxidase*

The function of glutathione S-transferase has implications in cell growth and oxidative stress as well as disease progression and prevention, which are present in subcellular compartments (e.g., cytosol, mitochondria, endoplasmic reticulum, nucleus, plasma membrane) [96]. Glutathione peroxidase (GPx), a selenoenzyme, plays a key role in the protection of organisms from oxidative damage by catalyzing the reduction of harmful hydroperoxides using thiol cofactors [97]. The function of GPx is to regulate hydroperoxide levels, but it might have dual roles [98,99]. The role of glutathione and glutathione-dependent enzymes in antioxidative processes is the maintenance and regulation of cell status, glutathionylation, and deglutathionylation, redox-dependent signaling, and apoptosis [100].

Emodin also demonstrates hepatoprotective effects against CCl_4-induced liver injury [101]. Emodin induces apoptosis in Dalton's lymphoma cells in association with the modulation of hydrogen peroxide-metabolizing antioxidant enzymes [102]. Emodin affects the mitochondrial capacity of ATP generation and antioxidant components as well as susceptibility against ischemia-reperfusion injury in rat hearts, although there is a sex difference [103]. Emodin also reportedly demonstrates antioxidant actions *in vivo* [104] and myocardial protective effects [105].

3.9. *Carcinogenesis*

Novel functions of emodin have been reported, namely that emodin enhances the repair of UV- and cisplatin-induced DNA damage and might even promote nucleotide excision repair (NER) capabilities in human fibroblast cells (WI38) [106] and human tongue cancer SCC-4 cells following DNA damage and the inhibition of DNA repair genes [107]. Emodin also demonstrates a proven ability to inhibit mutagenicity and the formation of 1-nitropyrene–induced DNA adducts in *Escherichia coli* PQ37 [108].

3.10. *Gene expression*

Several studies have reported that emodin affects the gene expression of human breast carcinoma BCap-37 cells [109] and downregulates the expression of transient receptor potential vanilloid 1 (TRPV1) ion channel protein mRNA and its functions in Dorsal root ganglion (DRG) neurons *in vitro*, thereby inhibiting inflammatory stimuli-induced hyperalgesia [110]. Emodin-mediated cytotoxicity in human lung adenocarcinoma H1650 (CRL-5883), human bronchioloalveolar carcinoma A549, lung squamous cell carcinoma H520, and H1703 cells is suppressed by Excision repair cross-complementary 1 (ERCC1) and Rapid Application Development (Rad)51 expression via extracellular regulated protein kinase 1/2 (ERK1/2) inactivation [111]. It has also been reported that emodin induces DNA damage and inhibits the expression of DNA repair genes in human tongue cancer SCC-4 cells [107]. Studies also show that emodin induces toxicological effects to the murine testicular gene expression profile [112] and inhibits the cytotoxic actions of tumor necrosis factor [113]. On the other hand, it has also been reported that emodin inhibits the migration and invasion in human tongue cancer SCC-4 cells due to the inhibition of the gene expression of matrix metalloproteinase (MMP)-9 [114].

3.11. *Glutathione S-transferase P expression*

Glutathione S-transferase P (GSTP) has been reported to regulate the S-glutathionylation of specific clusters of main proteins; it also plays a negative modulating role in some kinase pathways through ligand or protein interactions. GSTP is ubiquitously expressed in human tissue [115] and is linked to two cell-signaling functions critical to survival. It can sequester and negatively regulate c-jun N-terminal kinase (JNK) [116]. Catalytic reversal of S-glutathionylation is well characterized, but the role of GSTP in catalyzing the forward reaction contributes to the glutathionylation cycle [116].

Emodin reportedly induces neuroprotective effects in rat cortical neurons against β-amyloid–induced neurotoxicity [117]. Emodin induces apoptosis via an ROS-dependent mitochondrial signaling pathway in human lung adenocarcinoma A549 cells [118]. Emodin inhibits invasiveness, suppresses MMP-9 expression through the suppression of AP-1 and NF-κB in human cancer HSC5 cells (skin squamous cell carcinoma) and MDA-MB-231 cells (human breast cancer cell line) [119]. Likewise, emodin effectively suppresses hyaluronic acid (HA)-induced matrix metalloproteinase (MMP) secretion and the invasion of glioma through the inhibition of focal adhesion kinase (FAK), extracellular regulated protein kinase (ERK)1/2, and Akt/protein kinase B (PKB) activation and the partial inhibition of the transcriptional activities of activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) [33].

3.12. *Angiogenesis*

Therapeutic antiangiogenesis is widely viewed as a useful approach for the treatment of cancer, cardiovascular diseases, bone fractures, rheumatoid arthrities, and other diseases [120]. In tumor formation, angiogenesis plays a vital role in development, reproduction, and wound repair. Many studies describe natural and synthetic compounds with antiangiogenic activities, attracting notice to their potential applications in cancer prevention and treatment [121]. Emodin reportedly inhibits tumor-associated angiogenesis through the inhibition of ERK phosphorylation [122] and inhibits vascular endothelial growth factor-A–induced angiogenesis by blocking receptor-2 (KDR/Flk-1) phosphorylation [69]. Vascular endothelial growth factor (VEGF) has been studied for its role as a stimulant in angiogenesis and vascular permeability. Several studies show that emodin and its anthraquinone derivatives inhibit the angiogenesis and proliferation [123] of primary cultured bovine aortic endothelial cells in the absence or presence of basic fibroblast growth factor (bFGF) or the presence of VEGF in a dose-dependent manner [124,125]. Likewise, emodin inhibits VEGF receptors in human colon cancer cells [126], upregulates urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1, and promotes wound healing in human fibroblasts [127]. Emodin has been used in cancer therapies for the treatment of autoimmune diseases with anti-VEGF or anti-VEGFR (receptor) effects [69,126]. It has also been reported that emodin induces antiproliferative and antimetastatic effects in human pancreatic cancer SW1990 cells [128]. In human neuroblastoma SH-SY5Y cells, emodin inhibits the level of MMP, thus inhibiting migration and invasion *in vitro* [129].

3.13. *Drug resistance*

The overexpression of multidrug resistance (MDR) in tumor cells poses a serious obstacle to successful chemotherapy [130]. Treating cancer with chemotherapeutic agents and radiation leads to complications, such as the development of tumor resistance to therapy (radio- or chemoresistance). Emodin might sensitize tumor cells to radiation therapy and chemotherapeutic agents by inhibiting the pathways that lead to treatment resistance. Emodin has also been found to protect against therapy-associated toxicities [131]. Emodin induces the mechanisms that involve the ROS-mediated suppression of MDR and HIF-11 [60]. Our studies demonstrate emodin's cytotoxic and protective effects in rat C6 glioma cells: the survival effects involve Mdr1a, MRP2, MRP3, MRP6, and NF-κB [132]. Emodin may be involved in reducing the glutathione level and downregulating MDR-related protein 1 (MRP1) expression in gallbladder SGC996 cancer cells. In tumor-bearing mice, it has also been indicated that co-treatment with emodin/cisplatin suppresses tumor growth *in vivo* by increasing cancer cell apoptosis and downregulating MRP1 expression [61,133].

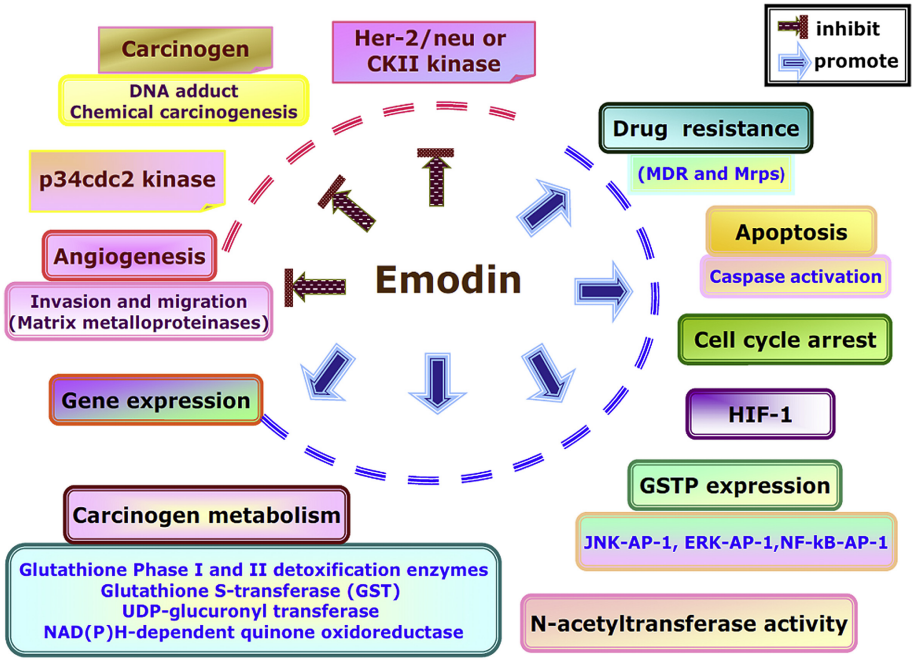

Fig. 2 – The pharmacology of emodin as a possible anti-cancer therapy.

4. Conclusion

Despite the fact that TCM research has been greatly accelerated with the advent of new technologies, we still need to work hard to gain stronger evidence that confirms the clinical applications of herbal medicines. Based on our observations and the results of previously reported studies, emodin can act as an anticancer agent against many human cancer cell lines through its effects across multiple signaling pathways. Over these past several years, our laboratory has evaluated agents that affect cell cycle arrest, apoptosis, metastasis, and angiogenesis in human cancer cell lines, both *in vitro* and *in vivo*, in addition to tumor cell growth, invasion, migration, and metastasis that are also involved in angiogenesis. Based on these observations regarding the effects of emodin, these findings may offer information that could be used in the design of novel therapeutic agents that inhibit tumor cells. Accordingly, we also summarize the pharmacology of emodin as a possible anticancer agent (Fig. 2).

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Review article

The silver bullet for cancer prevention: Chemopreventive effects of carotenoids

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ABSTRACT

Cancer has been a leading cause of death in many countries. Chemoprevention of various types of human cancer using dietary nutrients has received a lot of attention and interest in the past decade. Recently, carotenoids have been shown to prevent tumor growth and progression. Carotenoids demonstrated chemopreventive capability by interrupting several stages of cancer including initiation, promotion, progression, and metastasis. The molecular mechanisms of actions are through the modulation of cell-signaling pathways and gene expression. The results of our study suggested that carotenoids could act as chemopreventive agents against the growth and progression of human cancer cells.

1. Introduction

Carotenoids are organic compounds naturally occurring in plants and photosynthetic organisms such as algae [1,2]. Present reports suggest that >600 carotenoids have been identified. However, as a result of selective uptake in digestive tract, only 14 carotenoids with their metabolites have been identified in human plasma and peripheral tissues [3]. Carotenoids are commonly divided into two major classes, namely, carotenes and xanthophylls [4]. The presence of these carotenoids has been reported in fruits and vegetables. Some of the common carotenes are lycopene, carotene (α, β, γ, δ), and phytoene. On the other hand, the most common xenophylls are cryptoxanthin (α, β), zeaxanthin, lutein, violaxanthin, and astaxanthin [4]. Because of their colorful nature, carotenoid-rich foods such as fruits and vegetables always attract people's interest and induce their appetite. Results of many studies indicated that diets rich in vegetables and fruits can reduce the risk of several chronic diseases including cancer, cardiovascular diseases, and diabetes [5,6]. Many phytochemicals and nutrients present in these plant foods, such as carotenoids, antioxidant vitamins, polyphenols, folate, plant sterols, indoles, and fibers, contribute to the risk reduction [5,6]. Among these phytochemicals, carotenoids have been studied widely because of their beneficial effects on the human tissues and the diverse options they provide in improving human health. In humans, some carotenoids, such as β-carotene and β-cryptoxanthin, are precursors of vitamin A. Besides β-carotene, various carotenoids show more potent activity to suppress the process of carcinogenesis. In this

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review, we will mainly discuss the chemopreventive effects of β-carotene, lycopene, and cryptoxanthin.

2. Carcinogenesis

Cancer is an important public health issue worldwide. It has been a leading cause of death in many countries [7]. During tumorigenesis, accumulation of multiple gene mutations would lead to the neoplastic transformation of a single cell. These aberrant mutations or overexpression of several important genes contribute to the initiation of cancer and progression of human malignancies. Neoplastic transformation commonly affects three major classes of genes: proto-oncogenes, tumor-suppressor genes, and DNA repair genes. Several cellular proto-oncogenes have been activated through mutation. For example, the *ras* proto-oncogenes were a typical subset of gene mutation. Because of neoplastic transformation, *ras* (oncogene) genes were activated and exhibited transforming properties. The mutations of *ras* augment the activity of guanosine-5′-triphosphatase and transmission of signals to *raf*. Furthermore, mutations of the *raf* genes could also drive the mitogen-activated protein kinase (MAPK) signaling pathway and induce tumor growth and progression [8]. Several oncoproteins such as RAS, RAF, MAPK, and phosphatidylinositol-3 kinase (PI-3 K), Akt are frequently mutated in cancer [9,10]. Meanwhile, PI-3 K mutations could activate Akt and mTOR cascades to enhance cell survivals and escape from cell apoptosis. Suppression of apoptotic pathways involving the downstream caspase-3 molecule could avoid cell death [11]. RAS, RAF, and PI-3 K mutations occur in many cancer patients. The incidence of these cancer-specific point mutations is particularly high in many types of cancer and has been linked to poor outcomes. The aberrant activation of RAS/RAF/MEK/MAPK signaling pathways stimulate key processes involved in tumor growth and progression, including proliferation, angiogenesis, invasion, and metastasis [8]. The activation of MAPK/extracellular signal–regulated kinase (ERK) signaling pathway could induce the expression of cyclooxygenase-2 (COX-2) protein, its principal metabolite prostaglandin E_2, and inflammatory response [12,13]. More than 50% of colorectal carcinomas have elevated levels of COX-2 protein. The aberrant activation of MAPK/ERK signaling pathway also plays an important role in the disassembly of E-cadherin adherens complex and augments nuclear accumulation of β-catenin transcription factors in several types of cancer. Abnormal accumulation of β-catenin is correlated with tumor growth and progression [14–16]. A recent study indicated that β-catenin could be an important biomarker of human cancer. During the activation of these signaling pathways, upregulation of cell cycle–related protein, such as cyclin D1, is strongly correlated with tumor growth.

During the progression of tumor, overexpression of matrix metalloproteinases (MMPs) is highly correlated with inflammatory response, tumor growth, angiogenesis, and metastasis [17]. MMPs could degrade extracellular matrix and create a microenvironment that could support tumor development [18]. Invasion of cancer cells into the surrounding stroma occurs through the augmented expression of MMPs [19]. Previous studies suggested that elevated expression of MMP-9 was strongly correlated with poor prognosis and low survival rate in cancer patients [20,21]. However, suppression of MMPs could prevent the development of tumor [22].

3. Chemopreventive effects of carotenoids

3.1. *β-Carotene*

β-Carotene has been shown to inhibit the proliferation of cancer cells by their antioxidant activity or by their conversion into vitamin A. Surprisingly, previous human studies demonstrated that high doses of β-carotene (20 mg/day) supplementation enhanced the prevalence of lung cancer, especially in current smokers or people exposed to asbestos [23]. In order to clarify these controversial findings, scientists conducted several *in vitro* and *in vivo* studies. Many conclusive results showed that high levels of β-carotene in the smoke-exposed animals were prone to have plenty of oxidative metabolites of β-carotene, which enhance the metabolism of retinoic acid followed by diminished retinoid signaling, and induced cell proliferation. These findings suggest that dietary intake of β-carotene is still beneficial to induce chemopreventive effects. However, overdose of β-carotene in smokers would induce the formation and growth of lung cancer. Furthermore, these findings attracted more attention to the study of carotenoids. In this review, more evidences will be provided to demonstrate whether the remaining carotenoids are capable of preventing tumor growth.

3.2. *Lycopene*

Results of various epidemiological studies indicated that dietary intake of lycopene-rich tomatoes and tomato products is correlated with lower risk of cancer [24,25]. Serum and tissue levels of lycopene are also inversely correlated with the risk of several types of cancer. To further understand the chemopreventive effects, several studies have been conducted to investigate the molecular actions of lycopene. Most noticeably, one of the studies indicated that lycopene supplementation (at doses of 1.1 and 4.3 mg/kg body weight/day) could inhibit the proliferation of lung squamous cancer cells by the induction of apoptosis and upregulation of insulin-like growth factor–binding protein-3 in cigarette smoke–exposed ferrets [26]. Moreover, recent studies suggested that lycopene effectively inhibited the proliferation of several types of cancer by different mechanisms. Lycopene (at doses of 2, 5, and 10 μM) significantly inhibited the proliferation of colon cancer cells *in vitro* [27]. The molecular mechanisms of action were through the suppression of proliferative PI-3 K/Akt signaling cascades and augmented apoptotic pathways. Moreover, intake of lycopene (at doses of 3 and 6 mg/kg body weight/day) inhibited tumor growth in a mouse xenograft model of colorectal cancer [28]. Lycopene could also stabilize the expression of adherent E-cadherin molecules in colon cancer cells. Moreover, concomitant consumption of lycopene and eicosapentaenoic acid could synergistically inhibit the proliferation of colon cancer cells [29]. Huang et al. showed that lycopene significantly inhibited the proliferation and

metastasis of hepatoma cancer cells by the reduction of MMP-9 and vascular endothelial growth factor (VEGF) molecules [30]. The molecular mechanisms of action were achieved by the suppression of nuclear factor-kappa B (NF-κB p65) and stimulating protein-1 [31]. These findings made people feel confident in taking carotenoids as chemopreventive agents. Furthermore, recent studies demonstrated that lycopene can be converted into apo-10′-lycopenals by carotene-9′, 10′-oxygenase in both *in vitro* and *in vivo* conditions. The cleaved apo-10′-lycopenals can be further converted into apo-10′-lycopenoic acid and apo-10′-lycopenol in liver and lung tissues. The major metabolite of lycopene, apo-10′-lycopenoic acid, effectively inhibited the proliferation of lung cancer cells *in vitro* and *in vivo* conditions [32].

3.3. *Fucoxanthin*

Fucoxanthin is an orange-pigmented carotenoid found in seaweed. Because of its distinct structure, fucoxanthin belongs to the group of non-provitamin A carotenoids. However, fucoxanthin is an excellent free radical quencher under anoxic conditions. As free radicals and oxidative stress are involved in the initiation stage of cancer development, nutritional studies have focused on the antioxidant activity of fucoxanthin in the prevention of cancer development in the past few years. Many studies suggested that fucoxanthin can effectively inhibit or prevent the proliferation of several types of cancer cell lines such as prostate cancer, leukemia, and colorectal cancer cells [33,34]. The molecular mechanisms of fucoxanthin were probably through the induction of cell cycle arrest, apoptosis, and even by the expression of gap junction molecules in these cell lines. Moreover, fucoxanthin can inhibit the expression of antiapoptotic molecules such as Bcl-2 and Bcl-xl proteins. Yu et al. showed that fucoxanthin (50 and 75 μM) inhibited the proliferation of human gastric adenocarcinoma MGC 803 cells. The results demonstrated that fucoxanthin induced cell cycle arrest at G_2/M phase by the suppression of cyclin B1 protein. In addition, fucoxanthin also induced cell apoptosis by the suppression of JAK/STAT signaling pathway [35].

3.4. *β-Cryptoxanthin*

The structure of β-cryptoxanthin is similar to that of β-carotene. Under the action of carotene monooxygenase, cleavage of β-cryptoxanthin can lead to the formation of retinol and retinoic acid. Therefore, β-carotene and β-cryptoxanthin are provitamin A carotenoids. Epidemiological studies indicated that high intake of β-cryptoxanthin is associated with reduced risk of lung cancer, especially for current smokers. In the *in vitro* study, β-cryptoxanthin significantly inhibited the proliferation of lung cancer cells. β-Cryptoxanthin exhibited its anticancer effects by the upregulation of retinoic acid receptor-β and by the transactivation of retinoic acid response element–driven promoter activity. Supplementing dose-dependent β-cryptoxanthin with lycopene prevents lung inflammation by suppressing the levels of tumor necrosis factor-α and squamous metaplasia in lung tissues in cigarette smoke–exposed animals [36]. Moreover, β-cryptoxanthin suppressed the levels of oxidative damage to DNA, 8-OHdG, the activation of NF-κB, and expression of activator protein 1 (AP-1) [36]. These results suggest that β-cryptoxanthin might play an important role in protecting the lung tissue from smoke-induced inflammation, DNA damage, and squamous metaplasia in experimental animals.

3.5. *Astaxanthin*

Astaxanthin is a marine carotenoid without vitamin A activity. Results from an earlier study of xenograft tumor mouse model demonstrated that pretreatment of astaxanthin (0.005% astaxanthin for 8 weeks) suppressed the growth of mammary tumor in BALB/c mice [37]. Mice fed with astaxanthin before tumor initiation had increased blood levels of natural killer cells and plasma levels of γ-interferon compared with those fed with control diet (i.e., without astaxanthin). Such an effect was not observed in mice fed with astaxanthin after the tumor initiation. This study suggests that adequate blood astaxanthin is essential to protect against tumor initiation. Other evidences showed that astaxanthin could play important roles in the suppression of tumor invasion and progression. An earlier study showed that astaxanthin acted as a chemopreventive agent against 1,2-dimethyl hydrazine (DMH)–induced rat colon carcinogenesis. When administered with DMH (40 mg/kg body weight, subcutaneously), control group of experimental animals had high expression of NF-κB p65, COX-2, MMP-2, MMP-9, proliferating cell nuclear antigen, protein kinase B (Akt), and ERK-2. However, the treatment group that received astaxanthin (15 mg/kg of body weight/day, orally) had lower tumor size and reduced levels of these tumor biomarkers. Furthermore, astaxanthin induced apoptosis in colorectal carcinoma tissues of DMH-induced rats. The effects were associated with increased expression of caspase-3 protein in those astaxanthin-fed mice [38]. These results suggest that astaxanthin acts as a chemopreventive agent against tumor growth, invasion, inflammation, and progression.

3.6. *Lutein and zeaxanthin*

Lutein and zeaxanthin have been demonstrated as strong antioxidants and are widely distributed in vegetables and fruits. Epidemiological studies indicated that high intake of lutein/zeaxanthin could reduce the risk of variety of cancers including lung and colon cancer [39,40]. Although the clear molecular mechanism of lutein and zeaxanthin has not been studied well yet, several studies already revealed their chemopreventive effects in animals. Dietary supplementation of lutein also reduces colon carcinogenesis in carcinogen (DMH)-treated animals [41]. The chemopreventive effects of lutein against colon cancer were by the suppression of k-Ras and β-catenin expression and by the activation of protein kinase B.

4. Conclusions

This review demonstrated the chemopreventive effects of carotenoids in different aspects (Fig. 1). Many evidences suggest that carotenoids play important roles in the prevention of tumor growth, invasion, metastasis, and progression. Carotenoids are widely distributed in fruits and vegetables. Daily consumption of fruits and vegetables can provide an excellent way to prevent tumorigenesis.

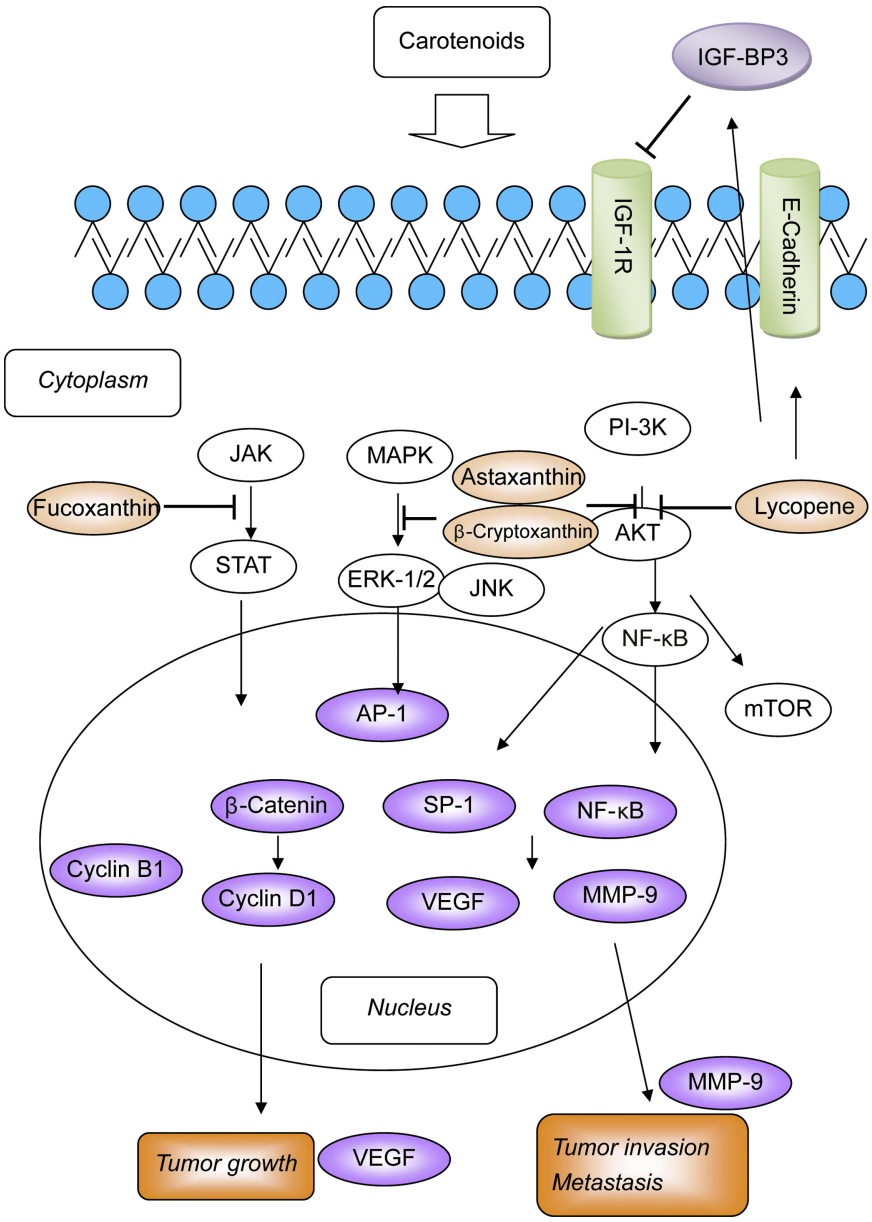

Fig. 1 – Proposed mechanisms of signaling pathways associated with carotenoids-mediated suppression of tumor growth and progression. ERK = extracellular signal–regulated kinase; IGF-1R = insulin-like growth factor 1; IGF-BP3 = insulin-like growth factor–binding protein-3; JAK = Janus kinase; JNK = c-Jun N-terminal kinase; MAPK = mitogen-activated protein kinase; MMP = matrix metalloproteinase; NF-κB = nuclear factor-kappa B; PI-3 K = phosphatidylinositol-3 kinase; SP-1 = stimulating protein-1; STAT = signal transducer and activator of transcription; VEGF = vascular endothelial growth factor.

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Original article

Clinical significance of circulating IL-10 and fibronectin levels in hepatocellular carcinoma patients with HBV infection

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ABSTRACT

Background/Introduction: Hepatocellular carcinoma (HCC) is the major cause of cancer-related death in Taiwan and is strongly associated hepatitis B virus (HBV) infections. Previous studies observed an imbalanced T-helper (Th)1/Th2 cytokine profile in HCC patients, however, less attention has been paid to the variation of Th2 cytokines, anti-inflammatory cytokines such as IL-4 and IL-10, in HCC patients. Increased expression of Fibronectin, VEGF and TGF-b1 in HCC patients has been observed, the relationship between these factors and other biomarkers remains unknown.

Purpose: This study examined the clinical significance of circulating interleukin-10 and fibronectin levels in HBV-infected hepatocellular carcinoma (HCC) patients.

Methods: HCC patients were classified according to international tumor-node-metastasis staging system as I ($n = 8$), II ($n = 24$), III ($n = 20$) and IV ($n = 10$). Thirty healthy subjects were included as control group.

Results: Compared with the control group, 7 test cytokines [interleukin (IL)-1, IL-2, IL-4, IL-6, IL-10, interferon-g and tumor necrosis factor (TNF)-a) were significantly higher in HCC patients ($p < 0.05$). Plasma TNF-a concentration in HCC patients increased from stage to stage ($p < 0.05$), while concentrations of both IL-4 and IL-10 decreased from Stage II to Stage IV ($p < 0.05$). HCC patients also had significantly higher plasma levels of VEGF, TGF-b1 and fibronectin than the control group ($p < 0.05$). Within HCC groups, both vascular endothelial growth factor (VEGF) and fibronectin levels decreased in Stage IV. VEGF, transforming growth factor-b1 (TGF-b1) or fibronectin were negatively correlated with IL-10, and the correlation coefficients were lower than e0.7. Both VEGF and TGF-b1 were positively correlated with fibronectin, and the correlation coefficients were higher than 0.7.

Conclusion: The circulating levels of IL-10 and fibronectin may reflect progression of HCC. Thus, monitoring these biomarkers may benefit HCC progression evaluation.

1. Introduction

Liver cancer, also called hepatocellular carcinoma (HCC), is the most common malignancy in the world [1]. In Taiwan, HCC is the major cause of cancer-related death [2], and is strongly associated hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infections [3,4]. Thus, virus infection is an important variable in clinical pathological investigation of HCC.

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Table 1 – Mean ± SD baseline characteristics in HCC patients and healthy control group.

Parameters	Control	HCC			
		I	II	III	IV
	$n = 30$	$n = 8$	$n = 24$	$n = 20$	$n = 10$
Body mass index (kg/m^2)	25.1 ± 2.6	24.5 ± 2.2	23.8 ± 1.7	21.2 ± 2.5	20.3 ± 1.6
Albumin (g/dL)	4.41 ± 0.43	4.17 ± 0.37	3.69 ± 0.51§	3.19 ± 0.67§	3.04 ± 0.73§
Creatinine (mg/dL)	0.74 ± 0.18	1.10 ± 0.29	1.08 ± 0.25	1.23 ± 0.19	1.34 ± 0.26
Uric acid (μmol/L)	210.3 ± 19.4	276.5 ± 22.5§	358.4 ± 40.7§	369.3 ± 52.4§	377.5 ± 34.0§
Bilirubin (mg/dL)	0.49 ± 0.13	0.92 ± 0.21	1.26 ± 0.31	2.29 ± 0.27§	3.31 ± 0.43§
α fetoprotein (ng/L)	25.1 ± 4.2	690.4 ± 170.2§	4872.5 ± 434.7§	7421.0 ± 653.8§	7038.2 ± 559.2§
Associated diseases					
Diabetes	2	1	2	1	0
Renal insufficient	0	0	2	1	1
Hypertension	3	1	0	0	1

§Means significantly different from control group, $p < 0.05$.

An imbalance between T-helper (Th)1 and Th2 cytokines has been observed in HCC patients [5,6]. The increased expression of several proinflammatory cytokines such as interleukin (IL)-6 and Th1 cytokines such as IL-1 in HCC patients has been reported [6,7]. The elevation of these cytokines means HCC deterioration including tumor growth and metastasis [7,8]. So far, less attention has been paid to the variation of Th2 cytokines, anti-inflammatory cytokines such as IL-4 and IL-10, in HCC patients. Since Th2 cytokines possess anti-inflammatory activity, the alteration of these cytokines may also affect HCC progression. Fibronectin is an extra cellular matrix glycoprotein, the expression of which is increased in liver tumor growth [9]; and increased fibronectin has been linked to resistance to therapy [10]. Apparently, fibronectin plays an important role in cancer progression, and is thus hypothesized to be highly associated with HCC progression. It is noted that most information regarding inflammatory stress of HCC is obtained from malignant tumors via surgical process. It may be more practical and feasible if the clinical information associated with inflammation and anti-inflammation, or the so-called Th1/Th2 cytokine profile, of HCC patients could be obtained from circulation via blood sampling.

Vascular endothelial growth factor (VEGF) is an angiogenic factors responsible for tumor angiogenesis: VEGF expression in tumor tissue is correlated with early metastasis spread and poor prognosis [11,12]. Transforming growth factor-β1 (TGF-β1) is highly expressed in many malignant tumors including HCC [13]. Although increased expression of VEGF and TGF-β1 in HCC patients has been observed, the relationship between these two factors and other biomarkers remains unknown.

Clinically, HCC patients could be classified according to the international tumor-node-metastasis (TNM) staging system [2]. The major purpose of this study was to examine the variation of IL-4, IL-10, fibronectin, VEGF, and TGF-β1 in HBV infected HCC patients classified by TNM staging. These results will enhance the understanding about inflammation variation presented in HCC patients.

2. Materials and methods

2.1. *Patients and healthy individuals*

This study protocol was approved by Ethical Committee of the Medicine Faculty at Chung Shan Medical University. Sixty-two patients with HBV infection and cytologically or histologically confirmed liver cancer at Chung Shan Medical University Hospital between May 2005 and October 2006 were included in

Table 2 – Mean ± SD plasma concentrations of two proinflammatory cytokines (IL-6, TNF-α), three Th1 cytokine (IL-1, IL-2, IFN-γ), and two Th2 cytokines (IL-4, IL-10) in healthy control group and HCC patients at different TNM stage.[1]

	Control	HCC			
		I	II	III	IV
IL-6, pg/mL	19.2 ± 3.6^a	135.3 ± 10.1^b	240.8 ± 21.5^c	332.7 ± 29.2^d	316.4 ± 32.5^d
TNF-α, pg/mL	23.8 ± 4.5^a	165.7 ± 16.3^b	257.6 ± 25.4^c	370.5 ± 34.8^d	454.7 ± 37.6^e
IL-1, pg/mL	19.4 ± 6.2^a	89.6 ± 10.5^b	180.5 ± 23.4^c	223.7 ± 28.6^c	245.7 ± 23.4^c
IL-2, pg/mL	21.3 ± 6.7^a	76.8 ± 13.2^b	151.7 ± 20.6^c	198.6 ± 23.8^c	160.5 ± 15.7^c
IFN-γ, pg/mL	18.0 ± 4.8^a	84.0 ± 9.3^b	177.4 ± 14.9^c	238.0 ± 22.5^d	263.2 ± 27.8^d
IL-4, pg/mL	18.7 ± 5.4^a	134.9 ± 18.3^c	156.3 ± 21.7^c	97.1 ± 9.2^b	80.6 ± 9.4^b
IL-10, pg/mL	20.5 ± 8.5^a	140.5 ± 15.7^c	245.2 ± 17.4^d	166.8 ± 11.6^c	71.3 ± 8.5^b

$^{a-e}$Means in a row without a common letter differ, $p < 0.05$.

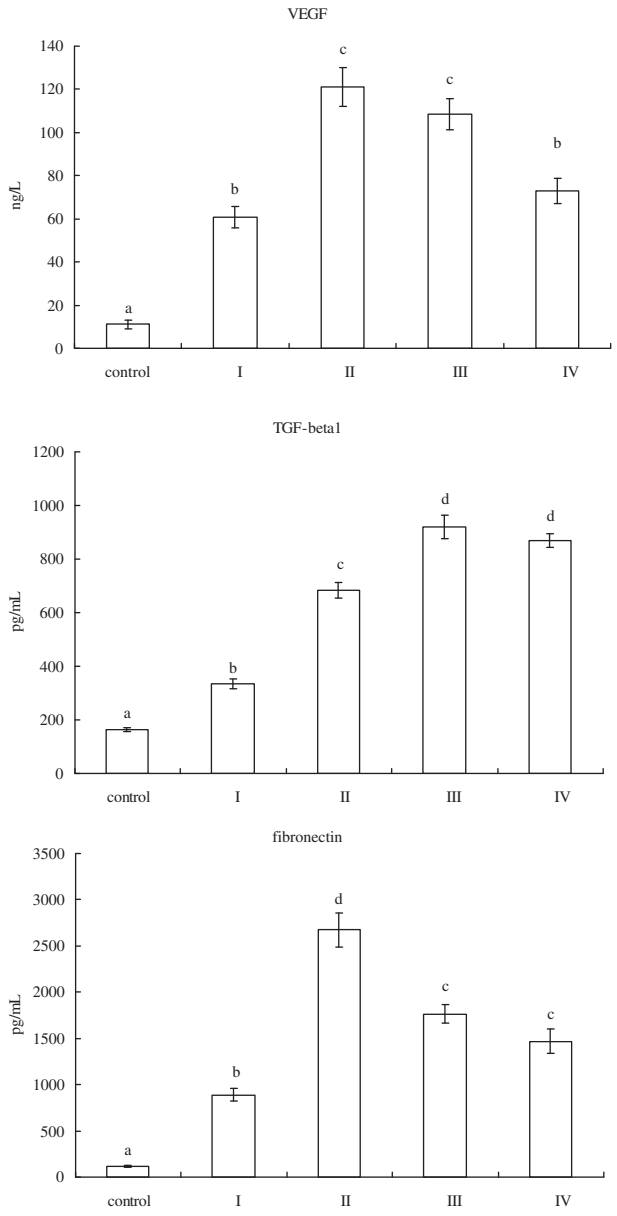

Fig. 1 – Mean ± SD plasma level of vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1) and fibronectin in healthy control group and HCC patients at different TNM stage. [a-d]Means among bars without a common letter differ, $p < 0.05$.

this study. Chronic HBV infection was confirmed by the presence of serum hepatitis B virus surface antigen (HBsAg), hepatitis B virus extracellular antigen (HBeAg) and HBV DNA. HBsAg and HBeAg were measured by radioimmunoassay (Abbott Laboratories, Chicago, IL, USA) and electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN, USA), respectively. Patients infected with HCV, and those with habitual alcohol intake, any other liver diseases (alcohol-, drug-, or obesity-induced liver disease, autoimmune hepatitis, hemochromatosis, α-1 anti-trypsin deficiency, Wilson disease or cirrhosis) were excluded. Patients with serum creatinine >15 mg/L, absolute neutrophil count $<1 \times 10^9$/L, platelet count $<50 \times 10^9$/L or hemoglobin <100 g/L were also excluded. These patients were aged 37 to 80 years (mean 64.1 years), were taking no therapy, and were newly diagnosed. HCC patients were classified according to the TNM staging system. The clinicopathological characteristics of these 62 HCC patients are shown in Table 1. Thirty healthy control participants (17 male, age 47–82 years, mean 61.3 years) were also included for comparison.

2.2. Blood sampling and biochemical measurements

Informed consent for study participation was obtained from 62 HCC patients and 30 healthy control subjects. A 15 mL peripheral blood sample was drawn from each participant after an overnight fasting. Plasma was separated from erythrocyte immediately after blood collection. Plasma levels of IL-1, IL-2, IL-4, IL-6, IL-10, interferon (IFN)-γ and tumor necrosis factor (TNF)-α were measured by ELISA using cytoscreen immunoassay kits (BioSource International, Camarillo, CA, USA). Samples were run in duplicate, and according to the manufacturer's instructions. The sensitivity of assay with the lower limit was 5 nmol/L for IL-1, IL-2, IL-4, IL-6, IL-10 and 10 nmol/L for IFN-γ and TNF-α. Plasma TGF-β1 and VEGF levels were measured by commercial ELISA kit (Quantikine Human VEGF, R&D System, Minneapolis, MN, USA). The sensitivity of assay with the lower limit was 5.0 ng/L, the intra-assay and interassay variabilities were 6.7% to 5.1% and 8.8% to 6.2%, respectively.

2.3. Statistical analysis

Each measurement was analyzed from 62 liver cancer patients and 30 healthy controls. All data presented in this study are mean ± SD. Data were subjected to analysis of variance (ANOVA) and differences with $p < 0.05$ were considered to be significant. Correlations between two variables

Table 3 – Correlation coefficients among IL-1, IL-2, IL-4, IL-10, VEGF, fibronectin, and TGF-β1 in 62 HCC patients.

	IL-1	IL-2	IL-4	IL-10	VEGF	fibronectin	TGF-β1
IL-1	1.000	0.483	−0.464	−0.545	0.570	0.528	0.493
IL-2		1.000	−0.425	−0.624	0.584	0.605	0.414
IL-4			1.000	0.672	−0.636	−0.715*	−0.702*
IL-10				1.000	−0.749*	−0.827*	−0.811*
VEGF					1.000	0.784	0.773*
Fibronectin						1.000	0.841*
TGF-β1							1.000

*Means $p < -0.05$.

were calculated by simple regression analysis (Minitab Inc., State College, Philadelphia, USA).

3. Results

As shown in Table 1, HCC patients had lower albumin, higher uric acid, and higher α fetoprotein concentrations in plasma than the control group ($p < 0.05$). Bilirubin level in HCC patients at Stages III and IV was significantly increased ($p < 0.05$). Plasma levels of cytokines in HCC patients are shown in Table 2. Compared with the control group, the concentrations of 7 test cytokines were significantly higher in HCC patients ($p < 0.05$). Plasma TNF-α concentration in HCC patients increased from stage to stage ($p < 0.05$). Both IL-4 and IL-10 levels were decreased from Stage II to Stage IV ($p < 0.05$). Plasma levels of VEGF, TGF-β1 and fibronectin from healthy control group and HCC patients at different TNM stage are presented in Fig. 1. HCC patients had significantly higher plasma concentrations of VEGF, TGF-β1 and fibronectin than the control group ($p < 0.05$). Within HCC groups, both VEGF and fibronectin concentrations decreased in Stage IV. The relationships among test factors in HCC patients is shown in Table 3. VEGF, TGF-β1, and fibronectin were negatively correlated with IL-10, with a correlation coefficient lower than −0.7. Both VEGF and TGF-β1 were positively correlated with fibronectin, and the correlation coefficient was higher than 0.7.

4. Discussion

The increased expression of several inflammatory cytokines such as IL-6 and TNF-α in HBV related liver cancer development has been reported previously [14]. Our present study further found that the release of Th1 and Th2 cytokines including IL-1, IL-2, IL-4, and IL-10 in circulation was markedly increased in HCC patients, which supported that inflammation and imbalance between Th1 and Th2 cytokines were involved in HBV-associated HCC deterioration.

It is known that IL-4 and IL-10 are anti-inflammatory immunomodulatory cytokines because they can induce expression of the IL-1R antagonist, and down-regulate the production of proinflammatory cytokines from human monocytes [15]. Moreover, IL-4 has a direct inhibitory effect on the development of human Th1 cells, and IL-10 is able to prevent Th1 effector function by reducing long-lasting T cell responsiveness [16,17]. Thus, the observed IL-4 and IL-10 increase in HCC patients at early stages (I+II) in our present study implied that the host self-defense system tended to suppress the inflammation reaction or to maintain cytokine balance. However, the overwhelming inflammation occurring in the late stages of liver cancer lowered IL-4 and IL-10 production, which suggested that the host's self-protection capability was diminished. Furthermore, the reduced IL-4 and IL-10 expression might indirectly favor expression of Th1 cytokines, which in turn exacerbated imbalance between Th1 and Th2 cytokines. It has been indicated that IL-4 is a potent inhibitor of hepatocyte growth factor, and may retard invasion and metastasis of carcinoma cells [18]. Thus, the variation of circulating IL-4 and IL-10 levels could be considered as predicators for evaluating host self-defense capability, liver immune function, and/or cancer progression.

Tumor angiogenesis is essential for solid tumorigenesis, growth, invasion and metastasis [19]. The elevation of circulating VEGF level indicated a promotion of tumor angiogenesis because VEGF benefited cancer cells spreading into normal liver parenchyma [19,20]. TGF-β1 could stimulate the metastatic capacity of tumor cells, and thus has been considered as a predictor for poor survival in HCC patients [21,22]. In the present study, TGF-β1 and VEGF levels in HCC patients were elevated, which supported that both were indicators to reflect HCC progression. Fibronectin could activate focal adhesion kinase, increase matrix metalloproteinase expression and promote cancer cell invasion and/or migration [23,24]. We found that circulating VEGF and fibronectin levels were dramatically reduced in patients at Stage IV. Although the mechanism remains unknown, it is possible that patients at the final cancer stage lost their capability to synthesize these molecules because of liver malfunctions. Further large scale clinical study is necessary to confirm the role of VEGF and fibronectin in HCC deterioration. In addition, we noted that VEGF, TGF-β1 and fibronectin levels in circulation were negatively correlated with IL-10 and IL-4. These relationships imply that the increased production of VEGF, TGF-β1, and fibronectin impaired the host's anti-inflammatory protection, and enhance inflammatory reactions. These findings indicate the clinical significance of these biomarkers in HCC progression. Plasma concentrations of IL-4, IL-10, VEGF, TGF-β1, and fibronectin are not routinely measured for HCC patients, at least in Taiwan. Clinical physicians and researchers should consider measuring these factors to assist clinical evaluation for HCC patients.

In conclusion, this clinical study provided several novel findings regarding the variation in the circulating levels of IL-4, IL-10, VEGF, TGF-β1 and fibronectin in HBV-infected HCC patients at different stages. The reduction of circulating IL-4 and IL-10 levels implies that patients loose their self-defense capability. Fibronectin profile might reflect HCC deterioration. Thus, monitoring these molecules in HCC patients might benefit diagnosis and/or prediction.

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Clinical Spotlight

Asymptomatic pulmonary nodule in a patient with early-stage lung adenocarcinoma—What is your diagnosis?

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A 56-year-old man with right lower lung adenocarcinoma (pT1aN0M0, stage Ia) had undergone right lower lung lobectomy in June 2007; no recurrence or metastasis was found during the follow-up period. As of June 2011, computed tomography (CT) of the chest (Fig. 1) revealed nodular opacity (2.6 cm × 2.3 cm) in parabronchial region of the right lung. The patient had no associated symptoms such as fever, productive cough, or chest pain. Level of carcinoembryonic antigen, a tumor marker, was within normal limits. In our early-stage lung cancer patient with pulmonary nodules who had received lobectomy, possibility of lung cancer recurrence was considered. He subsequently underwent radial endobronchial ultrasound (EBUS)-guided transbronchial needle aspiration (TBNA) of the parabronchial nodule (Fig. 2). Cytology of TBNA specimen (Fig. 3) revealed many yeast-form fungi encapsulated within epithelioid cells. Later serology test for *Cryptococcus* antigen was found to be positive, with a titer of 1:8; no HIV antibodies were detected. We did not examine cerebrospinal fluid due to low titer of the *Cryptococcus* antigen and absence of symptoms in the central nervous system infection. The patient received antifungal therapy with fluconazole (daily 400 mg) for 3 months, after which serum *Cryptococcus* antigen titer decreased to zero. In September 2010, a follow-up chest CT revealed shrinkage of the pulmonary nodule to a fibrotic band (Fig. 4).

Differentiating between benign lesion and primary tumor or metastasis in patients with pulmonary nodules is crucial for clinicians and difficult in some cases, such as in cancer patients. Surgical resection is the only recommended treatment for early-stage non-small-cell lung cancer. In early-stage lung cancer, after surgical resection, patients with pulmonary nodules may be regarded as having cancer relapse and given immediate chemotherapy. *Cryptococcus* is an opportunistic infection that predominantly affects immunocompromised patients. Approximately one-third of immunocompetent patients with *Cryptococcus* infection are asymptomatic; most

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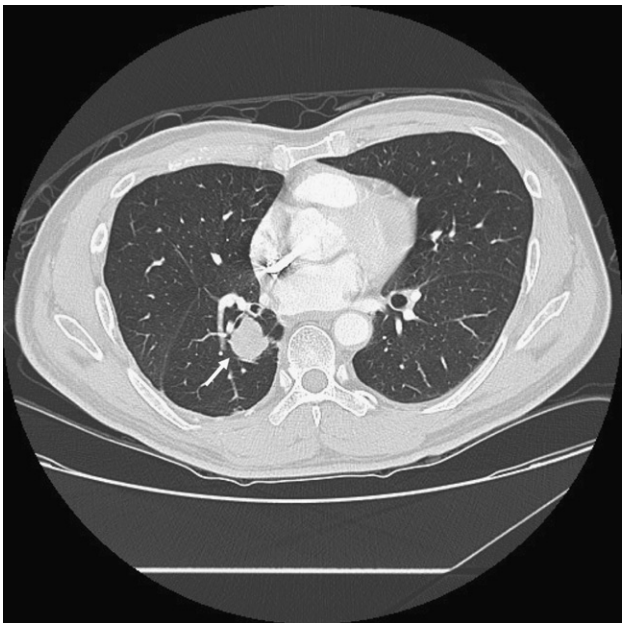

Fig. 1 – Computed tomography of the chest showing an ill-defined, soft-tissue nodule (white arrow) in the parabronchial region of the right lung.

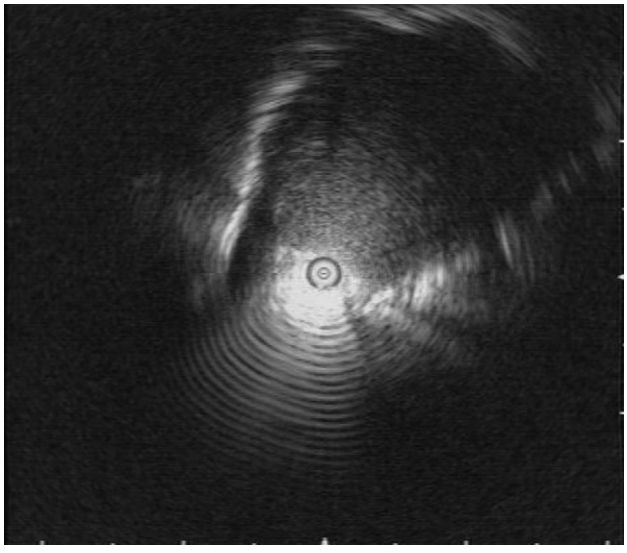

Fig. 2 – EBUS image of parabronchial pulmonary nodule. EBUS = endobronchial ultrasound.

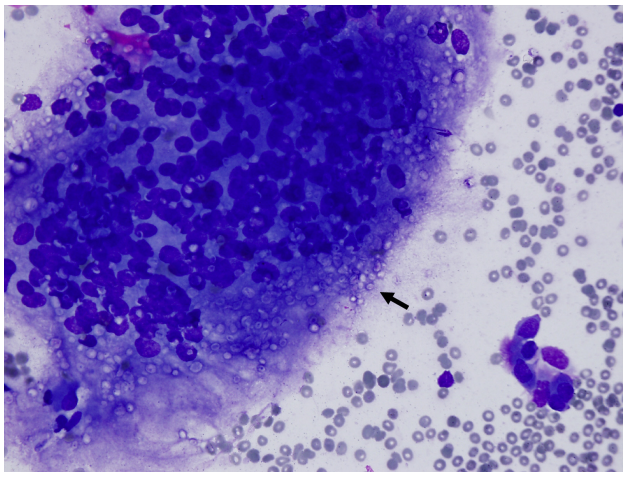

Fig. 3 – Cytology of EBUS TBNA specimen shows encapsulated forms of *Cryptococcus* (arrow), as demonstrated by Liu stain (400×). EBUS = endobronchial ultrasound; TBNA = transbronchial needle aspiration.

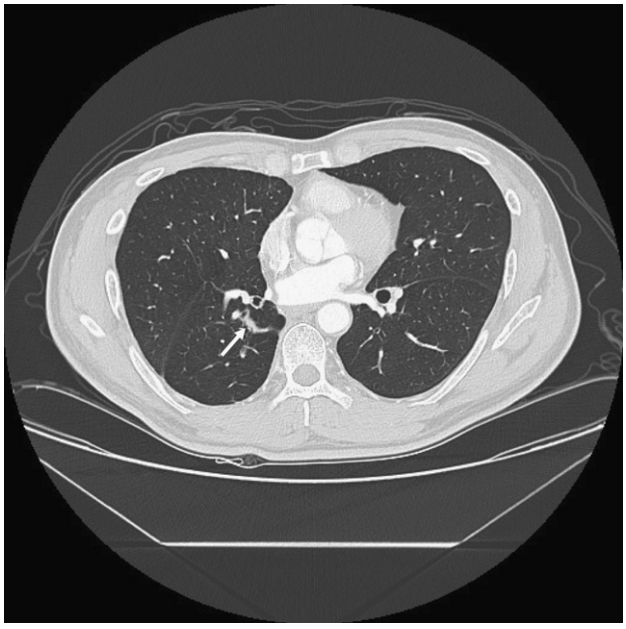

Fig. 4 – Computed tomography of the chest after 3 months of fluconazole treatment revealed shrinkage of the lung nodule to a fibrotic band (arrow).

common symptoms include cough, dyspnea, and fever. In asymptomatic patients, the pulmonary infection is usually discovered incidentally following chest radiography. We present the case of a lung cancer patient with single pulmonary nodule who underwent mini-invasive diagnostic method of EBUS TBNA to confirm the diagnosis of *Cryptococcus* infection. Differentiating between pulmonary *Cryptococcus* infection and tumor relapse in early-stage lung cancer is important for the correct management of the cancer. Biopsy must be performed for definite diagnosis and correct management of lung cancer patients with pulmonary nodules.

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For work involving experimental animals, the guidelines for their care and use should be in accordance with *European Commission Directive 86/609/EEC for animal experiments* (available at http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm); this should be stated in the methods section of the manuscript.

4. Reporting Clinical Trials

All randomized controlled trials submitted for publication should include a completed Consolidated Standards of Reporting Trials (CONSORT) flow chart (available at http://www.consort-statement.org). This Journal has adopted the proposal from the International Committee of Medical Journal Editors (ICMJE) that require, as a condition of consideration for publication of clinical trials, registration in a public trials registry. Purely observational studies (those in which the assignment of the medical intervention is not at the discretion of the investigator) do not require registration. Further information can be found at http://www.icmje.org.

5. Identification of Patients in Descriptions, Photographs and Pedigrees

A signed statement of informed consent to publish (in print and online) patient descriptions, photographs and pedigrees should be obtained from all subjects (parents or legal guardians for minors) who can be identified (including by the subjects themselves) in such written descriptions, photographs or pedigrees. Such persons should be shown the manuscript before its submission. Omitting data or making data less specific to de-identify patients is acceptable, but changing any such data is not acceptable.

6. Previous Publication or Duplicate Submission

Submitted manuscripts are considered with the understanding that they have not been published previously in print or electronic format (except in abstract or poster form) and are not under consideration in totality or in part by another publication or electronic medium.

7. Basic Criteria

Articles should be written in English (using American English spelling) and meet the following basic criteria: the material is original, the information is important, the writing is clear and concise, the study methods are appropriate, the data are valid, and the conclusions are reasonable and supported by the data.

8. Article Categories

8.1. *Review Articles*

These should aim to provide the reader with a balanced overview of an important and topical subject in the field, and should be systematic and critical assessments of literature and data sources. They should cover aspects of a topic in which scientific consensus exists as well as aspects that remain controversial and are the subject of ongoing scientific research. All articles and data sources reviewed should include information about the specific type of study or analysis, population, intervention, exposure, and tests or outcomes. All articles or data sources should be selected systematically for inclusion in the review and critically evaluated.

By invitation only. The format for review articles will be jointly decided by the Editors and the contributing author. Typical length: no more than 4000 words, 50–100 references.

8.2. *Original Articles*

These may be randomized trials, intervention studies, studies of screening and diagnostic tests, laboratory and animal studies, cohort studies, cost-effectiveness analyses, case-control studies, and surveys with high response rates, which represent new and significant contributions to the field.

Section headings should be: Abstract, Introduction, Methods, Results, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

The Introduction should provide a brief background to the subject of the paper, explain the importance of the study, and state a precise study question or purpose.

The Methods section should describe the study design and methods (including the study setting and dates, patients/participants with inclusion and exclusion criteria, or data sources and how these were selected for the study, patient samples or animal specimens used, explain the laboratory methods followed), and state the statistical procedures employed in the research.

The Results section should comprise the study results presented in a logical sequence, supplemented by tables and/or figures. Take care that the text does not repeat data that are presented in tables and/or figures. Only emphasize and summarize the essential features of any interventions, the main outcome measures, and the main results.

The Discussion section should be used to emphasize the new and important aspects of the study, placing the results in context with published literature, the implications of the findings, and the conclusions that follow from the study results. Typical length: no more than 5500 words, 40–80 references.

8.3. *Case Reports*

These are short discussions of a case or case series with unique features not previously described that make an important teaching point or scientific observation. They may describe novel techniques, novel use of equipment, or new information on diseases of importance. Section headings should be: Abstract, Introduction, Case Report, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

The Introduction should describe the purpose of the report, the significance of the disease and its specificity, and briefly review the relevant literature.

The Case Report should include the general data of the case, medical history, family history, chief complaint, present illness, clinical manifestation, methods of diagnosis and treatment, and outcome.

The Discussion should compare, analyze and discuss the similarities and differences between the reported case and similar previously reported cases. The importance or specificity of the case should be restated when discussing the differential diagnoses. Suggest the prognosis of the disease and possibility of prevention. Typical length: no more than 1500 words, 20–40 references.

8.4. *Short Communications*

These should be concise presentations of clinical or preliminary experimental results. Section headings should be: Abstract, Introduction, Methods, Results, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

Typical length: no more than 1000 words, 20–40 references, with no more than four figures or tables. The Editors reserve the right to decide what constitutes a Short Communication.

8.5. *Letters to the Editor*

Letters are welcome in response to previously published articles, and may also include interesting cases that do not meet the requirement of being truly exceptional, as well as other communications of general interest. Letters should have a title and include appropriate references, and include the corresponding author's mailing and e-mail addresses. Letters are edited, sometimes extensively, to sharpen their focus. They may be sent for peer review at the discretion of the Editors. Letters are selected based on clarity, significance, and space. Typical length: no more than 600 words, 5–10 references; 1 table and/or 1 figure may be included.

8.6. *Editorials*

Editorials are invited articles or comments concerning a specific paper in the Journal or a topical issue in the field. While normally invited, unsolicited editorials may be submitted. Typical length: no more than 1500 words, 15–30 references.

9. Manuscript Preparation

Text should be typed double-spaced on one side of white A4 (297 × 210 mm) paper, with outer margins of 2.5 cm. A manuscript should include a title page, abstract, text, acknowledgments (if any), conflicts of interest statement (if any), references, and figures and tables as appropriate. Each section of the manuscript should begin on a new page. Pages should be numbered consecutively, beginning with the title page.

9.1. *Title Page*

The title page should contain the following information (in order, from the top to bottom of the page):

- category of paper
- article title
- names (spelled out in full)* of all the authors, and the institutions with which they are affiliated; indicate all affiliations with a superscripted lowercase letter after the author's name and in front of the appropriate affiliation
- corresponding author details (name, e-mail, mailing address, telephone and fax numbers)

**The name of each author should be written with the family name last, e.g., Jing-Lin Chang. Authorship is restricted only to direct participants who have contributed significantly to the work.*

9.2. *Abstract and Keywords*

Abstracts should be no more than 300 words in length. Abstracts for Original Articles should be structured, with the section headings: Background/ Introduction, Purpose(s)/Aim(s), Methods, Results, Conclusion. Abstracts for Case Reports are unstructured, but should include the significance and purpose of the case presentation, the diagnostic methods of the case, the key data, and brief comments and suggestions with regard to the case. Abstracts for Review Articles and Short Communications should also be unstructured. No abstract is required for Letters to the Editor and Editorials. For the article categories that require an abstract, 3–5 relevant keywords should also be provided in alphabetical order.

9.3. *Main Text*

The text for Original Articles should be organized into the following sections: Background/ Introduction, Purpose(s)/Aim(s), Methods, Results and Discussion. Sections for Case Reports are: Introduction, Case Report, and Discussion. Each section should begin on a new page.

9.3.1. Abbreviations

Where a term/definition will be continually referred to, it must be written in full when it first appears in the text, followed by the subsequent abbreviation in parentheses. Thereafter, the abbreviation may be used. An abbreviation should not be first defined in any section heading; if an abbreviation has previously been defined in the text, then the abbreviation may be used in a subsequent section heading. Restrict the number of abbreviations to those that are absolutely necessary.

9.3.2. Units

Système International (SI) units must be used, with the exception of blood pressure values which are to be reported in mmHg. Please use the metric system for the expression of length, area, mass, and volume. Temperatures are to be given in degrees Celsius.

9.3.3. Names of drugs, devices and other products

Use the Recommended International Non-proprietary Name for medicinal substances, unless the specific trade name of a drug is directly relevant to the discussion. For devices and other products, the generic term should be used, unless the specific trade name is directly relevant to the discussion. If the trade name is given, then the manufacturer name and the city, state and country location of the manufacturer must be provided the first time it is mentioned in the text, for example, "…SPSS version 11 was used (SPSS Inc., Chicago, IL, USA)."

9.3.4. Statistical requirements

Statistical analysis is essential for all research papers except case reports. Use correct nomenclature of statistical methods (e.g., two sample *t* test, not unpaired *t* test). Descriptive statistics should follow the scales used in data description. Inferential statistics are important for interpreting results and should be described in detail.

All p values should be expressed to 2 digits to the right of the decimal point, unless $p < 0.01$, in which case the p value should be expressed to 3 digits to the right of the decimal point. The smallest p value that should be expressed is $p < 0.001$, since additional zeros do not convey useful information; the largest p value that should be expressed is $p > 0.99$.

9.3.5. Personal communications and unpublished data

These sources cannot be included in the references list but may be described in the text. The author(s) must give the full name and highest academic degree of the person, the date of the communication, and indicate whether it was in oral or written (letter, fax, e-mail) form. A signed statement of permission should be included from each person identified as a source of information in a personal communication or as a source for unpublished data.

9.4. *Acknowledgments and Conflicts of Interest Statement*

General acknowledgments for consultations, statistical analysis, etc., should be listed concisely at the end of the text, including the names of the individuals who were directly involved. Consent should be obtained from those individuals before their names are listed in this section. All financial and material support for the research and work from internal or external agencies, including commercial companies, should be clearly and completely identified. Ensure that any conflicts of interest (financial and/or non-financial) are explicitly declared.

9.5. *Abbreviation list*

A term that appears more than three times in a paper should be abbreviated. Spell out the term on first mention, followed by the abbreviated form in parentheses. Thereafter, please use the abbreviated form. Supply a list of nonstandard abbreviations used in the paper at the end of the main text, in alphabetical order, giving each abbreviation followed by its spelled-out version.

9.6. *References*

9.6.1. In the main text, tables, figure legends

- References should be indicated by numbers in square brackets in line with the text, and numbered consecutively in order of appearance in the text.
- References cited in tables or figure legends should be included in sequence at the point where the table or figure is first mentioned in the main text.
- Do not cite uncompleted work or work that has not yet been accepted for publication (i.e., "unpublished observation", "personal communication") as references. Also see Section 9.3.5. above.
- Do not cite abstracts unless they are the only available reference to an important concept.

9.6.2. In the references section

- References should be limited to those cited in the text and listed in numerical order, NOT alphabetical order.
- References should include, in order, author surnames and initials, article title, abbreviated journal name, year, volume and inclusive page numbers. The last names and initials of all the authors up to 6 should be included, but when authors number 7 or more, list the first 6 authors only followed by "et al". Abbreviations for journal names should conform to those used in MEDLINE.
- If citing a website, provide the author information, article title, website address and the date you accessed the information.

- Reference to an article that is in press must state the journal name and, if possible, the year and volume.

Authors are responsible for the accuracy and completeness of their references and for correct text citation.

Examples are given below.

Standard journal article
Chen Z, Fan M, Bian Z, Zhang Q, Zhu Q, Lu P. Immunolocalization of heat shock protein 70 during reparative dentinogenesis. Chin J Dent Res 2000;3:50–5.

Journal supplement
Kaplan NM. The endothelium as prognostic factor and therapeutic target: what criteria should we apply? J Cardiovasc Pharmacol 1998;32(Suppl 3):S78–80.

Journal article not in English but with English abstract
Nakayama H, Ishikawa T, Yamashita S, Fukui I, Mutoh T, Hikichi K, et al. CSF leakage and anosmia in aneurysm clipping of anterior communicating artery by basal interhemispheric approach. No Shinkei Geka 2011;39:263–8. [In Japanese, English abstract]

Book
Bradley EL. Medical and surgical management. Philadelphia: Saunders; 1982, p. 72–95.

Book chapter in book with editor and edition
Greaves M, Culligan DJ. Blood and bone marrow. In: Underwood JCE, editor. General and systematic pathology. 4th ed. London: Churchill Livingstone; 2004, p. 615–72.

Bulletin
World Health Organization. World health report 2002: reducing risk, promoting healthy life. Geneva, Switzerland: World Health Organization; 2002.

Company/manufacturer publication/pamphlet
Eastman Kodak Company, Eastman Organic Chemicals. Catalog no. 49. Rochester, NY: Eastman Kodak; 1977, p. 2–3.

Electronic publications
Duchin JS. Can preparedness for biological terrorism save us from pertussis? Arch Pediatr Adolesc Med 2004;158:106–7. Available from: http://archpedi.ama-assn.org/cgi/content/full/158/2/106. Accessed June 5, 2004.

Smeeth L, Iliffe S. Community screening for visual impairment in the elderly. Cochrane Database Syst Rev 2002(2):CD001054. doi:10.1002/14651858.CD1001054.

Items presented at a meeting but not yet published
Durbin D, Kallan M, Elliott M, Arbogast K, Cornejo R, Winston F. Risk of injury to restrained children from passenger air bags. Paper presented at: 46th Annual Meeting of the Association for the Advancement for Automotive Medicine; September 2002; Tempe, AZ.

Greenspan A, Eerdekens M, Mahmoud R. Is there an increased rate of cerebrovascular events among dementia patients? Poster presented at: 24th Congress of the Collegium Internationale Neuro-Psychopharmacologicum (CINP); June 20–24, 2004; Paris, France.

Khuri FR, Lee JJ, Lippman SM. Isotretinoin effects on head and neck cancer recurrence and second primary tumors. In: Proceedings from the American Society of Clinical Oncology; May 31–June 3, 2003; Chicago, IL. Abstract 359.

Item presented at a meeting and published
Cionni RJ. Color perception in patients with UV- or blue-light-filtering IOLs. In: Symposium on Cataract, IOL, and Refractive Surgery. San Diego, CA: American Society of Cataract and Refractive Surgery; 2004. Abstract 337.

Material accepted for publication but not yet published
Carrau RL, Khidr A, Crawley JA, Hillson EM, Davis JK, Pashos CL. The impact of laryngopharyngeal reflux on patient-reported quality of life. Laryngoscope. In press.

Ofri D. Incidental findings: Lessons from my patients in the art of medicine. Boston, MA: Beacon Press. In press.

Theses and dissertations
Undeman C. Fully automatic segmentation of MRI brain images using probabilistic diffusion and a watershed scale-space approach [master's thesis]. Stockholm, Sweden: NADA, Royal Institute of Technology; 2001.

Ayers AJ. Retention of resin restorations by means of enamel etching and by pins [dissertation]. Indianapolis: Indiana University; 1971.

Website
American Association of Oral and Maxillofacial Surgeons. Wisdom teeth. AAOMS Web site. http://www.aaoms.org/wisdom_teeth.php. Published January 23, 2008. Updated March 9, 2009. Accessed November 15, 2009.

9.7. Tables

Tables should supplement, not duplicate, the text. They should have a concise table heading, be self-explanatory, and numbered consecutively in the order of their citation in the text. Information requiring explanatory footnotes should be denoted using superscripted lowercase letters in alphabetical order (a, b, c, etc.). Asterisks (*, **) are

used only to indicate the probability level of tests of significance. Abbreviations used in the table must be defined and placed after the footnotes. If you include a block of data or table from another source, whether published or unpublished, you must acknowledge the original source.

9.8. *Figures*

9.8.1. General guidelines

The number of figures should be restricted to the minimum necessary to support the textual material. They should have an informative figure legend and be numbered in the order of their citation in the text. All symbols and abbreviations should be defined in the legend. Patient identification should be obscured. All lettering should be done professionally and should be in proportion to the drawing, graph or photograph. Photomicrographs must include an internal scale marker, and the legend should state the type of specimen, original magnification and stain.

Figures must be submitted as separate picture files at the correct resolution (see Section 9.7.2. below). The files should be named according to the figure number, e.g., "Article1_Fig1", "Article1_Fig2".

9.8.2. Formats

Regardless of the application used, when your electronic artwork is finalized, please "save as" or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

- EPS: Vector drawings. Embed the font or save the text as "graphics".
- TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
- TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
- TIFF: Combination of bitmapped line/half-tone (color or grayscale): a minimum of 600 dpi is required.
- DOC, XLS or PPT: If your electronic artwork is created in any of these Microsoft Office applications, please supply "as is".

Please do not:

- Supply files that are optimized for screen use (like GIF, BMP, PICT, WPG); the resolution is too low;
- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

A detailed guide on electronic artwork is available at http://www.elsevier.com/artworkinstructions.

10. The Editorial and Peer Review Process

As a general rule, the receipt of a manuscript will be acknowledged within 1 week of submission, and authors will be provided with a manuscript reference number for future correspondence. If such an acknowledgment is not received in a reasonable period of time, the author should contact the Editorial Office.

Submissions are reviewed by the Editorial Office to ensure that it contains all parts. The Editorial Office will not accept a submission if the author has not supplied all the material and documents as outlined in these author instructions.

Manuscripts are then forwarded to the Editor-in-Chief, who makes an initial assessment of it. If the manuscript does not appear to be of sufficient merit or is not appropriate for the Journal, then the manuscript will be rejected without review.

Manuscripts that appear meritorious and appropriate for the Journal are reviewed by at least two Editorial Board members or expert consultants assigned by the Editor-in-Chief. Authors will usually be notified within 6 weeks of whether the submitted article is accepted for publication, rejected, or subject to revision before acceptance. However, do note that delays are sometimes unavoidable.

11. Preparation for Publication

Once a manuscript has been accepted for publication, the authors should submit the final version of the manuscript in MS Word format, with all tables/figures as applicable, to the Editorial Office.

Accepted manuscripts are copyedited according to the Journal's style and PDF page proofs are e-mailed by the Publisher to the corresponding author for final approval. Authors are responsible for all statements made in their work, including changes made by the copy editor.

12. Publication Charges and Reprints

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Only complete manuscript submissions will be considered for publication. Complete submission must include:

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☐ Authorship statement signed by all authors
☐ Signed conflicts of interest disclosure statement
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☐ 3–5 relevant keywords in alphabetical order: required for Review Article, Original Article, Case Report, Short Communication (MeSH terms are recommended; see http://www.ncbi.nlm.nih.gov/mesh?term)
☐ Main text
☐ References in the correct format, cited in numerical order, and all references in the List are cited in the Text/Tables/Figures, and vice versa

AND, where applicable

☐ Acknowledgments
☐ Conflicts of interest statement
☐ Table headings and tables, each on a new page
☐ Figure legends, on a new page
☐ Electronic picture files of all figures; resolution of 300 dpi for halftone images, 600 dpi for combination art (halftone + line art), and 1000 dpi for line art

Further considerations:

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☐ Color figures are clearly marked as being intended for: (I) color reproduction on the Web (free of charge) and in print; or (II) color reproduction on the Web (free of charge) and in grayscale in print (free of charge). If option (II), then grayscale versions of the figures are also supplied for printing purposes.

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Acknowledgments

All persons who have made substantial contributions to the work reported in the manuscript (e.g., technical help, writing and editing assistance, general support), but who do not meet the criteria for authorship, are named in the Acknowledgments and have given us their written permission to be named. If we have not included an Acknowledgments, then that indicates that we have not received substantial contributions from non-authors.

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