Review

Synergistic Effect of Extracellular Vesicles and Nanoquercetin As Anticancer Therapeutics in Hepatocellular Carcinoma

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Review

Synergistic Effect of Extracellular Vesicles and Nanoquercetin as Anticancer Therapeutics in Hepatocellular Carcinoma

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Abstract: Over the few decades, cancer-associated mortalities and morbidities were continuously increased worldwide despite sophisticated technological advancements. Pharmaceutical interventions associated with drugs exhibit a high degree of side effects and toxicities in addition to very high costs. Subsequently, to reduce or to vanish the side effects and high costs, researchers are now exploring natural bioactive compounds such as quercetin in its nanoformulations along with biologics as cargo delivery vehicles. Quercetin along with mesenchymal stromal lineage-derived extracellular vesicles (EVs) possesses an anti-cancer potential that can be explored to treat hepatocellular carcinoma (HCC). Exerting enhanced effect, nano quercetin synergistically with EVs triggers the anti-cancer mechanisms by regulating and dysregulating several signalling mechanisms including *NF-κB, p53, JAK/STAT, MAPK, Wnt/β*-catenin and *PI3K/AKT*, in addition to *PBX3/ERK1/2/CDK2*, and miRNAs modulation. In addition, findings regarding the potential checkpoints of anti-cancer signalling pathways were investigated that offer opportunities to develop engineered EVs incorporated with nano quercetin for the development of novel therapeutics to treat HCC in future. In this present mechanistic review, we abridged the regulation of such signalling mechanisms synergetic approach of nano quercetin and EVs. The regulatory role of EVs in the manifestation of innumerable miRNAs has also been tailed with special context to HCC.

Keywords: nanoquercetin; extracellular vesicles; hepatocellular carcinoma; therapeutics; anticancer; drug delivery

1. Background

Liver cancer or primary hepatic malignancy accounts for the sixth most common form of human cancer worldwide and among this 90% of the liver cancer cases exhibit hepatocellular carcinoma (HCC) [1,2]. The major risk factors for HCC include hepatitis B and C infections, fatty liver disease and excess alcohol intake [3]. Among these risk factors, hepatitis B virus infection is among the prominent risk factor for the development of HCC, which alone accounts for 50% of cases [4]. Unrelenting virological response (UVR) with the usage of antiviral drugs has significantly diminished the risk of HCC attributed to hepatitis C virus infection [5]. However, in the West, non-alcoholic steatohepatitis (NASH) attributed to metabolic disorders including diabetes mellitus and obesity is increasing at an alarming rate contributing to the aetiology of HCC [6,7]. Age is also considered to be the contributory risk factor in the progression of non-alcoholic fatty liver disease (NAFLD) related HCC. In one of the previously published studies, it was observed that patients with NAFLD-attributed HCC were more aged compared to virus-associated HCC [8]. Age-associated gut microbiota modulation in patients presenting NAFLD is also considered to be at high risk of developing HCC [9].

World Health Organization (WHO) considered liver cancer the prime cause of cancer-related mortalities worldwide. It is also estimated that in the year 2020, about 0.83 million people died due to it [10]. In Asia, HCC is among the most common form of liver cancer that accounts for 0.5 million

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deaths with 0.6 million new cases in 2020 [11,12]. Asian men demonstrated higher incidence and mortality compared to Asian women making it fourth highest incidence and second highest mortality. Moreover, among Asian women, liver cancer accounted seventh-highest incidence and sixth-highest mortality in the year 2020 [10]. A sharp decline in the average annual percent change (AAPC) in incidence rates was observed in cases of liver cancer in South Korea (-2.2%), Japan, China (-1.6%) and the Philippines (-1.7%) as documented by previously published study [12]. On the contrary, South-Western Asian countries including Israel showed a hike in AAPC [12]. In another report published by GLOBOCAN 2020, countries including Iran, Afghanistan, Qatar, Azerbaijan, Iraq, and Nepal also showed a worrying trend [13]. Studies demonstrated that eastern Asia, northern Africa and Micronesia are with highest incidence rates while the highest mortality rates were shown by eastern Asia, northern Africa and south-eastern Asia [14,15] (Figure 1, 2, and 3). With the increased incidence and mortality rates of HCC in several parts of the world, it is necessary to focus on such issues with newer technologies and interventions.

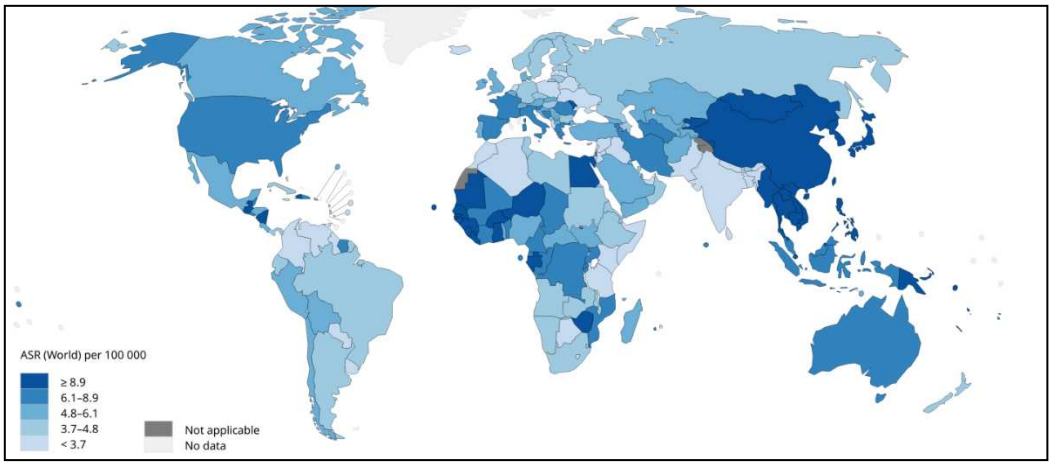

Figure 1. Estimated age-standardized incidence rates (ASR) (worldwide) for liver cancer, both sexes and all ages, in 2020. Data source: GLOBOCAN 2020. Map production: IARC (http://gco.iarc.fr/today) World Health Organization. (Copy Rights 2020). https://gco.iarc.fr/today/online-analysis-map?v=2020&mode=population&mode_population=continents&population=900&populations=900&key=asr&sex=0&cancer=11&type=0&statistic=5&prevalence=0&population_group=earth&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=10&group_cancer=1&include_nmsc=0&include_nmsc_other=0&projection=natural-earth&color_palette=default&map_scale=quantile&map_nb_colors=5&continent=0&show_ranking=0&rotate=%255B10%252C0%255D. (Ref [3]).

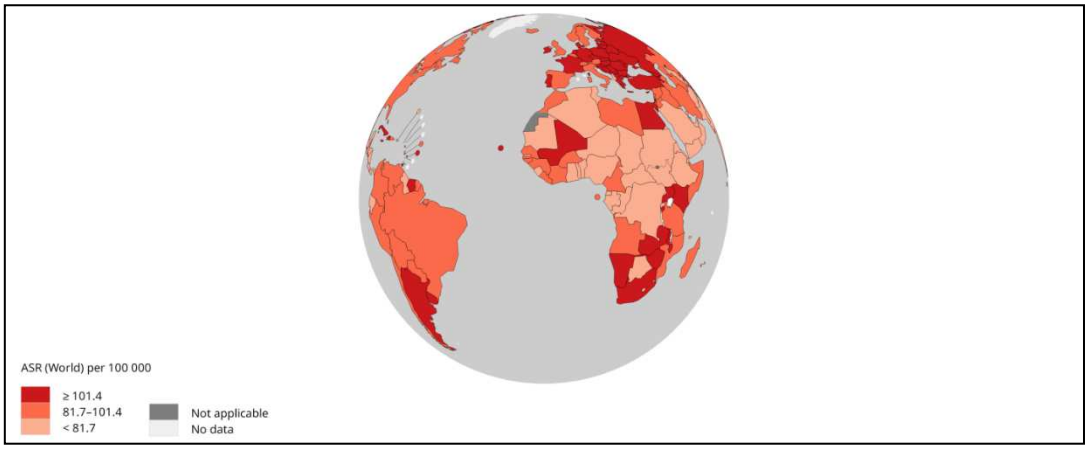

Figure 2. Estimated age-standardized mortality rates (worldwide) in 2020 for liver cancer, both sexes and all ages. Data source: GLOBOCAN 2020. Map production: IARC (http://gco.iarc.fr/today) World Health Organization. (Copy Rights 2020). https://gco.iarc.fr/today/online-analysis-

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map?v=2020&mode=population&mode_population=regions&population=250&populations=250&key=asr&sex=0&cancer=39&type=1&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=10&group_cancer=0&include_nmsc=0&include_nmsc_other=1&projection=globe&color_palette=default&map_scale=quantile&map_nb_colors=3&continent=0&show_ranking=0&rotate=%255B10%252C0%255D (Ref 3).

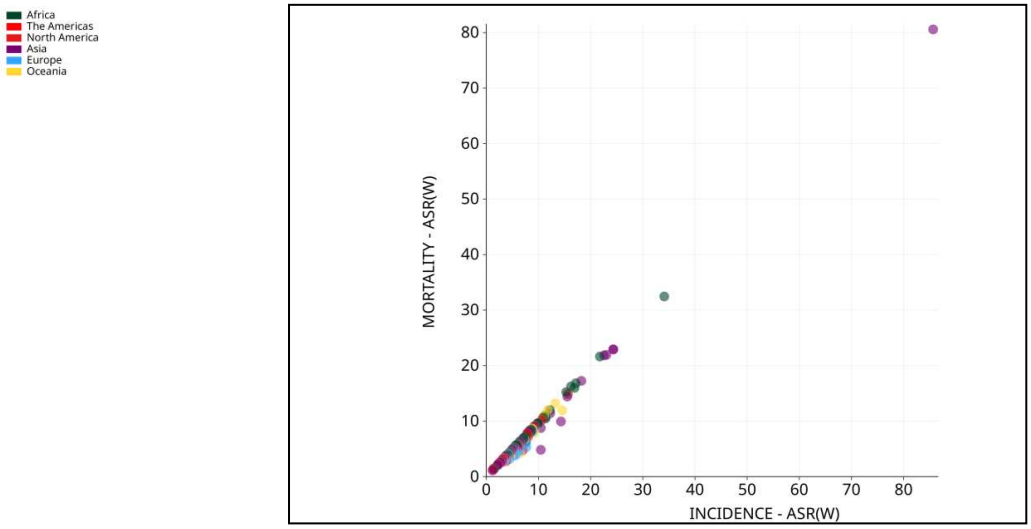

Figure 3. Mortality-ASR (worldwide) vs. Incidence-ASR (worldwide) in 2020, for both sexes and all ages. Data source: GLOBOCAN 2020. Map production: IARC (http://gco.iarc.fr/today) World Health Organization. (Copy Rights 2020). https://gco.iarc.fr/today/online-analysis-scatter-plot?v=2020&mode=cancer&mode_population=continents&population=900&populations=900&key=asr&sex=0&cancer=11_11&type=0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=5&group_cancer=0&include_nmsc=0&include_nmsc_other=1&fit_to_screen=1&same_scale=1&axis_indicators=%257B%2522x%2522%253A%2522inc%2522%252C%2522y%2522%253A%2522mort%2522%257D&axis_keys=%257B%2522x%2522%253A%2522asr%2522%252C%2522y%2522%253A%2522asr%2522%252C%2522log_scale_x%2522%253Afalse%252C%2522log_scale_y%2522%253Afalse%257D (Ref 3).

Several staging approaches were fabricated to classify HCC including Hong Kong Liver Cancer (HKLC), cancer of the liver Italian program (CLIP), Okuda, Barcelona clinic liver cancer (BCLC) (Figure 4), American Association for the Study of Liver Diseases (AASLD) [16,17]. Among these staging approaches, the latter two were widely used worldwide. Some other staging regimes involve the classification of HCC using molecular genetics, metabolism, immunological properties and chromosomal arrangements [18].

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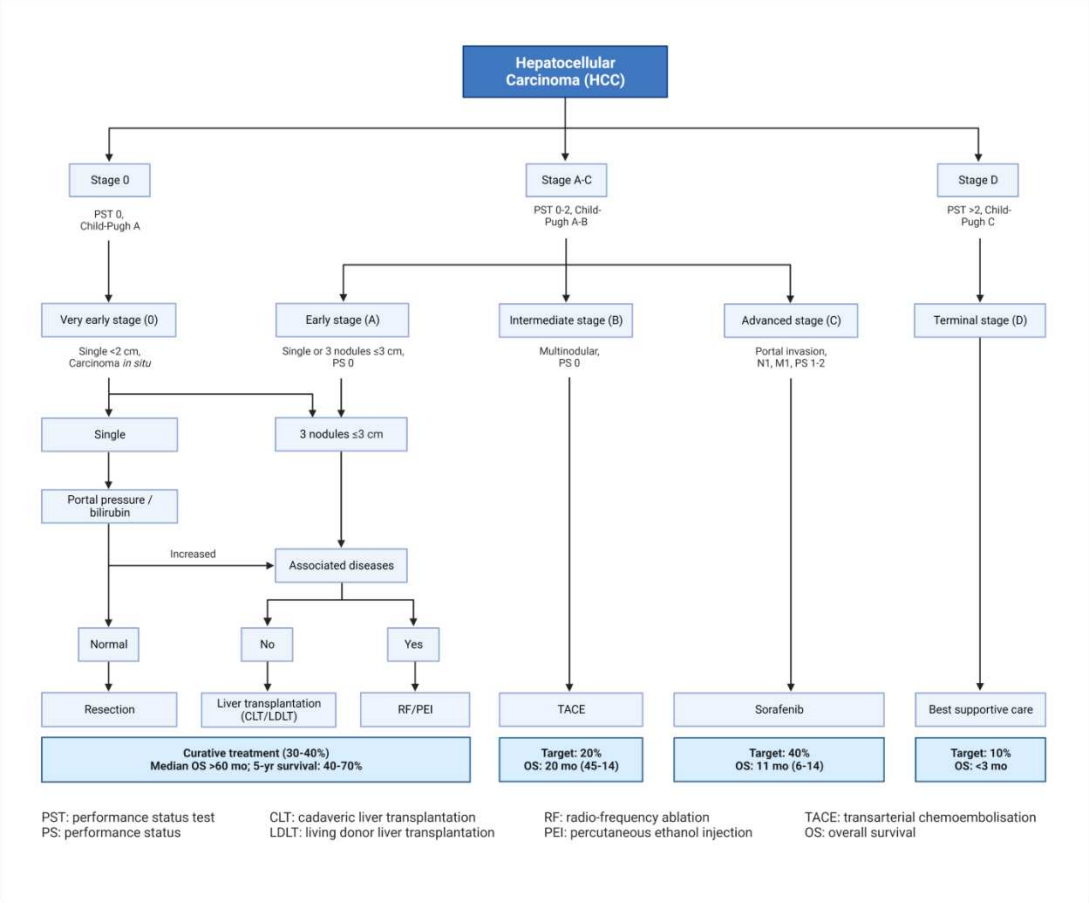

Figure 4. Barcelona Clinic Liver Cancer (BCLC) staging system.

Current interventional approaches for HCC include liver resection, transplantation, transarterial therapy, the implication of tyrosine kinase inhibitors (systemic therapy), and local ablative therapy. Moreover, along with conventional therapies, several drugs including sorafenib, lenvatinib, atezolizumab and bevacizumab along with chemotherapeutic agents such as doxorubicin (DOX) showed restricted effects along with associated extrinsic and intrinsic drug resistance [19].

Despite multiple pharmacological interventions, treatment of advanced-stage HCC does not fulfil the standard health outcomes. Such unsatisfied outcomes may be attributed to several reasons including drugs associated adverse effects, low bioavailability, high toxicity, non-specific delivery of pharmacological agents, high cost at large-scale production, immune complications, and anaphylactic responses [20]. To overcome such limitations, researchers are now looking to explore possibilities of using nanoparticles and extracellular vesicles (EVs) as drug delivery vessels for the treatment of almost every cancer. The present review will focus on exploring the potential of EVs and quercetin nanoparticles (Qnps) for the treatment of HCC. Moreover, the mechanistic pathways inhibiting the pathogenesis of HCC with Qnps and EVs are also explored. We further examined the evidence supporting the use of biological molecules including EVs and quercetin for treatment regimes for HCC. Finally, we performed a critical analysis of current clinically approved drugs/immunotherapies for HCC and ongoing preclinical/clinical trials using EVs and Qnps as anti-cancer agents in patients with HCC.

2. Pathophysiology of HCC

The pathophysiology of HCC exhibits complex multifactor mechanisms. HCC progression and hepatocytes' malignant transformation depend on the interplay between various factors including genetic predisposition, viral and non-viral elements, the severity of liver disease and the cellular microenvironment at its early stage. It was seen that nearly 80% of liver cirrhotic patients develop

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HCC attributed due to molecular alterations [2]. Viral elements include etiological infections associated with HCV and HBV while the non-viral elements include alcohol consumption, NASH, use of aflatoxin, tobacco and aristolochic acid has been identified as a trigger of cancer mechanisms in the liver [21]. In addition to aforesaid factors, some specific immune and molecular causes were identified as an initiator of HCC [21]. In this respect, studies of such molecular and immunological checkpoints are necessary to understand the onset, progression and treatment using biopharmaceuticals for these targets. Some of the major checkpoints were extensively studied elsewhere [2,22,23].

3. Molecular triggers of hepatocellular carcinoma

In patients with liver cirrhosis, the neoplasm advances through a sequential cascade of histopathological modulations ultimately initiating HCC. Histomorphological characteristics of HCC include highly vascularized tumours with prominent acinar and wide trabeculae along with loss of Kupffer cells and reticulin network [24]. In advanced HCC, tumours were found to be encapsulated with the presence of septae that are positive for CD34 and α-smooth muscle actin (SMA). Studies have found that mature hepatocytes are the primary cells responsible for HCC origin and progression in addition to liver stromal cells [23,25,26]. Studies in past demonstrated that repetitive stress to regenerating hepatocytes triggers genetic lesions that initiate transformation and oncogenesis progression [27]. The study observed that alterations in cyclin-A2 or E1 proteins of the cell cycle favour the progression of HCC, especially in non-cirrhotic patients which is further mediated by activation of E2F and ATR transcriptional pathways along with inactivation of RB1 and PTEN [27].

In patients with NASH, CD8+PD1+ T cells promote hepatocyte death and thereby favour the micro-environment for HCC pathogenesis and progression [28]. On contrary, somatic, genomic and epigenetic modulations also trigger the HCC. A study showed the single nucleotide polymorphisms (SNP) of PNPLA3 (rs738409), TM6SF2 (rs585542926) and HSD17B13 (rs72613567) predispose to liver carcinogenesis that increases the probability of HCC [29]. Genotoxic compounds including aflatoxin B1 and aristolochic acid (promote inversion of T to A) were known to trigger somatic mutations that again increased the risk of HCC progression [30].

4. Checkpoint targets of hepatocellular carcinoma

Hepatocellular carcinoma pathogenesis is triggered by several mechanistic pathways that involved numerous checkpoints and can be explored as targeted therapies in HCC. The following checkpoints were considered to play a pivotal role in HCC.

4.1. Wnt–β-catenin signalling

CTNNB1

A CTNNB1-related active mutation is a major canonical component of the Wnt signalling pathway and is exhibited in nearly 11-41 % of patients with liver cancer [32–34]. *CTNNB1* is actively involved in the synthesis of actin cytoskeleton responsible for halting cell division [35]. Indeed mutations of *CTNNB1* were reported to be significantly correlated with *TERT* promoter, *NFE2L2*, *MLL2*, *ARID2*, and *APOB* [36,37]. Studies related to human HCC found that *CTNNB1* mutations concurrently occurred with the upregulation of *Met, Myc, or Nrf2* [38–40]. Drugs including sorafenib and gamma-secretase inhibitors were also studied as effective targets indulging the *CTNNB1* mechanism [30,41].

Adenomatous Polyposis Coli (APC)

Human *APC* mutations originated within the central core region of the open reading frame (ORF) commonly known as MCR (mutation cluster region) that produces truncated proteins [42]. Moreover, this event triggers the loss of several factors including β-catenin binding sites (20R), nuclear localization sequences (NLS), axin binding sites (ABS) and C-terminal basic domain (CTBD),

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which are responsible for cytoskeletal interfaces. Sporadic *APC* mutations are considered to be the contributory factor for tumorigenesis. Mutations in *APC* significantly modulate the Wnt–β-catenin signalling, that in turn initiates the origin and progression of HCC.

AXIN1

AXIN1 mutations were found to be associated with nearly 5-19% of patients with liver cancer [32,33]. *AXIN1* negatively regulate the Wnt/β-catenin signalling by modulating the expression of β-catenin [43]. A study found that upregulated expression of wild-type *AXIN1* intimidated the cellular proliferation in HCC along with induction of programmed cell death, and thereby can be used as a molecular target to treat HCC [43]. In continuation with this study, another author used adenovirus-mediated gene transfer of *AXIN1* and initiated HCC cell apoptosis [44]. *AXIN* was found to be an inhibitor of tankyrase 1 and 2 through XAV 939 and hence can be used as a novel therapeutic target within Wnt signalling [45].

4.2. Telomere maintenance

TERT

TERT promoter mutations were known to be associated with the pathogenesis of HCC. It was reported by previous studies that *the TERT* promoter showed mutation at the upstream of ATG translation start site at positions -124 (G>A) and -146 (G>A) [46,47]. Mutations in *TERT* promoter sequences produce a de novo consensus binding region for the ETS (E-twenty-six) transcription factor that further triggers the increased production of TERT proteins that in turn attenuate the telomerase activity and length [48–50]. A recent study reported mutation of *the TERT* promoter in HCC patients at -297 (C>T) upstream of the ATG translational region generating an AP2 consensus sequence [51]. It has been found that the protein expressed by *RB/E2F* gene regulates the activity of the *TERT* promoter and contributed to liver cancer [52]. A past study also showed that *TERT* gene activation was triggered by the binding of RNA-binding fox-1 homolog 3 (RBFOX3) with AP2β that in turn activates telomerase and promotes HCC [53]. In another study, it was found that SP1 and YAP1 activate the TERT gene expression in the HepG2 cell line [54].

4.3. Cell cycle regulation

TP53

Nearly 13-48% of patients with liver cancer exhibit *TP53* mutations [32,33]. *TP53* gene suppresses the tumours by arresting the growth and apoptosis of cancerous cells [32]. A previously published study from West demonstrated that mutations in *the TP53* gene especially in patients with HCC are associated with poor health outcomes and prognosis [33]. In another study, it was found that non-inflamed tumours exhibit T-cell exclusion mediated either through *TP53* gene mutations also known as an intermediate class [55]. Authors of another study concluded that *TP53* mutations especially known as hot spot mutations at R249S and V157F were associated with poor outcomes and prognosis of patients with HCC [56].

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)/DR4/DR5

TRAIL receptor 2/DR5 is a member of the TNF receptor family and is associated with chromosome 8p21-22. The study has reported mutations of TRAIL-R2 in cancer [57]. A similar study detected single point mutation in the DR5 domain among 1% of HCC patients suggesting the importance in the pathogenesis of carcinogenesis [57]. It was found that TRAIL and IER3 proteins trigger the inhibition of Wnt/β-catenin signalling [58]. The study suggested that TRAIL/IER3/β-catenin axis plays important role in HCC and can be explored as a checkpoints or therapeutic target against HCC [58].

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DR4/5 clustering and oligomerization mediated by TRAIL protein recruit several adaptor factors to generate a death-inducing signalling complex (DISC) that in turn further activates the caspases-8 and 10 within this complex along with TRADD and RIP kinases [59–63].

CDKN2A, CCND1, FGF3, FGF4 or FGF19

It seems that nearly 8% of the HCC cases exhibit CDKN2A deletions mutation [36]. It was known that *CDKN2A* is also a tumour suppressor gene that triggers the arresting of the cell cycle at the G1 and G2 phases and can act as a potential checkpoint for HCC therapy. Moreover, it also inhibits the expression of *CDK4/6* and *MDM2*, which are responsible for oncogenic action [64]. A previous study reported that loss of *CDKN2A* in patients with HCC attenuates the rise of CDK4/6 inhibitors in the advanced stage [65].

In liver cancer, it was found that nearly 5-7% and 4-6% of the patients exhibit mutations of *CCND1* and *FGF3, FGF4*, or *FGF19* respectively [36,66]. It was studied that augmentation of CCND1, *FGF3, FGF4*, or *FGF19* in patients with resected HCC is associated with poor prognosis and outcome [36]. A plausible study showed suppression of 11q13.3 amplicon by anti-FGF19 antibody along with anti-sense RNA mediated knockdown of FGF19 or CCND1 [67].

4.4. Oxidative stress

Hepatocytes exhibit numerous fatty acids that trigger oxidative stress along with endoplasmic reticulum (ER) stress. Furthermore, these stresses cause cellular damage and inflammation [68]. In one of the animal studies, it was found that ER stress can cause NASH-triggered HCC due to the activation of several pathways including NF- κB and TNF [69]. One of the previously published studies suggested that ER stress is mediated through the activation of hepatosteatosis and secondly due to the promotion of SREBP1 activation and increasing the process of lipogenesis [69]. ER stress in association with steatosis generates ROS in hepatocytes that are the primary cause of oxidative stress and oncogenic mutations. These ROS triggers the lipotoxic death of hepatocytes and thereby activate the macrophages. Further release of TNF-α also triggers the activation of chemokines and growth factors that attenuate the inflammatory microenvironment of hepatocytes [69]. In addition, ROS production induces DNA damage due to mitochondrial dysfunction and hence contributes to the pathophysiology of HCC in humans [70].

In the previously published study, it was found that mTORC2 activations within the hepatocytes trigger the concentration of sphingolipid glucosylceramide and thereby increased ROS generation which in turn leads to HCC [71]. Impaired cholesterol metabolism also triggers the pathophysiology of HCC [72]. A clinical study demonstrated the trend of HCC in patients and found that NASH posed a higher risk for HCC pathogenesis compared to NAFLD [73].

5. Potential anti-cancer mechanism of nano quercetin in HCC

Quercetin belongs to the naturally occurring flavonoid class and is widely known for its therapeutic effect including pro-apoptotic, proliferative and antioxidant [74]. It is a well-known inhibitor of casein kinase-2α that is responsible for HCC pathogenesis [75]. Some studies also decipher the role of casein kinase-2α in the apoptosis mechanism and activation of death receptors pathways [76–78]. Moreover, studies showed that nanoformulation of quercetin improves the mechanistic action and therapeutic properties compared to pure quercetin form due to several limitations including less bioavailability, slow absorption and short action. Therefore, nano quercetin showed enhanced anti-cancer activities by significantly modulating the signalling pathways as shown below:-

5.1. Wnt/β-catenin signaling pathway

The wnt/β-catenin signalling pathway regulates several biological processes including cell differentiation, proliferation, migration, and APC/β-catenin/Tcf pathway [79]. In another in-vitro study, it was found that quercetin showed inhibition of SOX2, Nanog, and Oct4 expression along

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with β-catenin nuclear translocation that in turn resulted in downregulated expression of β-catenin-dependent transcriptional activity [80]. In another study, it was found that 20μM quercetin showed reduced viability through regulating DKK1, 2 and 3 proteins that in turn act as checkpoints of Wnt signalling [81].

5.2. PI3K/AKT pathway

PI3K mediates the translocation process of AKT to the plasma membrane and regulates the mechanism of cell cycle progression, differentiation, cell survival and cell proliferation [82]. PI3K/AKT is also observed to regulate the expression of Bax (Bcl-2 protein family member) which is responsible for the anti-apoptotic mechanism [83]. Authors from the previously published study reported the anti-cancer activity of quercetin against HCC1937 PTEN cancer cell lines through regulation of AKT/PKB phosphorylation [84].

In one of the studies, it was suggested that flavonoids directly or indirectly inhibit the mTOR signalling mechanism [85]. It is known that PDK1 is considered to be a major kinase necessary for the development of the mammalian cell. It is found that in the cancer microenvironment, the degree of phosphorylation of AKT kinase at Thr-308 was significantly increased [86]. Another study found that quercetin triggers the down-regulation of phosphorylation of PDK1 and is hence considered to be the therapeutic target of quercetin and regulatory checkpoints at Ser-473 and Thr-308 [87]. Quercetin is considered to be a broad-spectrum inhibitor of PI3K/AKT1/2 as found by a previous study [88]. Hence, it was considered that quercetin inhibits the AKT1/2 by acting directly by inducing Ser/Thr kinase activity and down-regulating the PI3K.

5.3. JAK/STAT signalling

JAK/STAT signalling mechanism regulates the immune microenvironment, cell death, proliferation, division and tumour growth. It is known that JAK/STAT pathway is controlled by ERK, MAPK and PI3 kinase. It was reported that carcinomas were associated with the deregulation of the JAK/STAT pathway [89]. Qin and their coworkers observed the role of quercetin on the JAK/STAT pathway and observed that MGC-803 cells were arrested at the G2/M stage of the cell cycle mediated through p-STAT3 signalling and also reduces the expression of leptin along with its corresponding receptors [90]. It was also reported that quercetin inhibits the IL-6-triggered glioblastoma cell migration, proliferation and growth by regulating the STAT-3 signalling mechanism mediated through reduced expression of GP130 and JAK1 [91].

Quercetin is known to modulate apoptosis through activation of caspase 3, 8 and PARP cleavage that enable the cell to arrest in the sub-G0/G1 phase of the cell cycle along with reduction of p-JAK1, MMP-9 and p-STAT3 expression [92]. Authors from previous studies claimed that quercetin regulates the apoptosis and autophagy mechanism through the expression of caspase-3 that is further inhibited by JAK2 along with cyclin D1 and mTOR that in turn suppressed STAT3/5 signalling mechanism [93,94]. Moreover, quercetin was found to show reduced proliferation potential of HCC along with an increased rate of cellular apoptosis due to regulation of the cell cycle through the expression of CyclinB1 protein [95]. CyclinB1 is a cell cycle protein which is synthesized in S and G2/M phases. Therefore it is believed that quercetin inhibits the cell cycle at the G2/M phase along with triggering apoptosis.

5.4. The MAPK signalling

Mitogen-Activated Protein Kinase (MAPK) exhibits three primary classes of kinases including ERKs, JNK/SAPK and p38s. It is considered that MAPK 14,7 and 12 regulate cellular proliferation, gene expression, differentiation, growth, mitosis and apoptosis [96]. In addition, one study done on SMMC7221 cells found that quercetin suppresses the proliferation, and reduces the lipopolysaccharide-initiated oxidation along with inhibition of the MAPK signalling pathway [97]. It was also found that quercetin significantly suppresses the activity of p38 MAPK in the fibrotic liver of rats [98]. It was found that isoquercetin activates the caspases 3, 8 and 9 that in turn significantly

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increases the apoptosis and triggers the JNK phosphorylation through suppression of ERK and p38 MAPK as shown in Figure 5 [99].

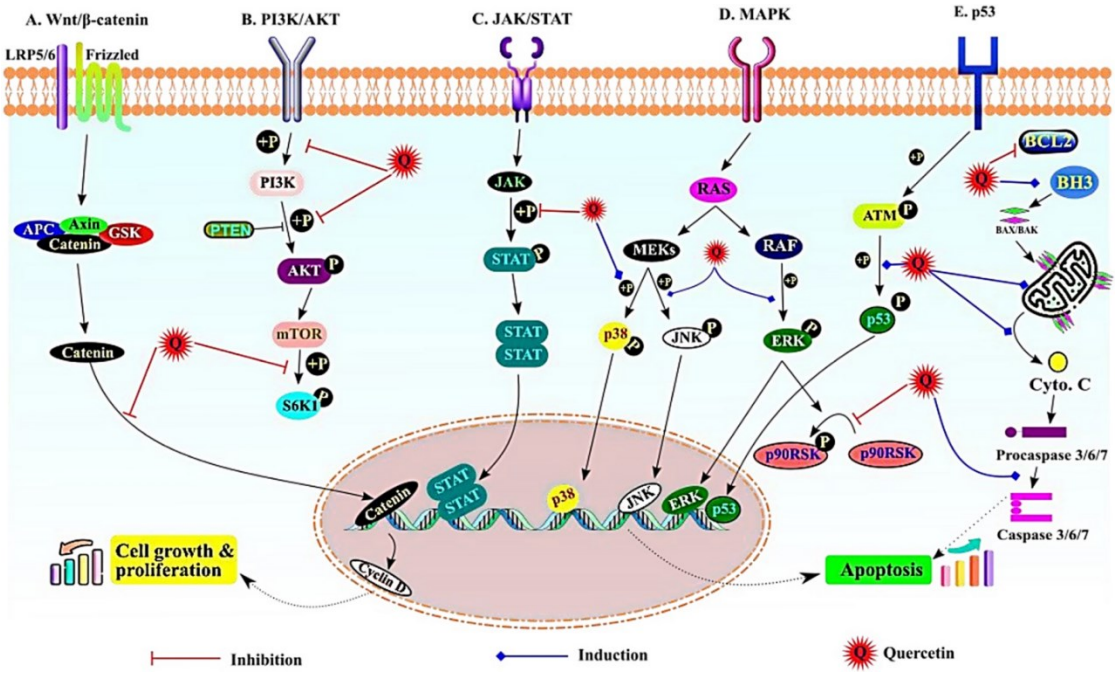

Figure 5. The most important signalling pathways affected by quercetin during cancer prevention. A) Wnt/β catenin pathway; quercetin inhibits β-catenin translocation to the nucleus, B) PI3K/Akt pathway; inhibition of phosphorylation of PI3k, Akt, and S6K, C) JAK/STAT pathway; inhibition of p-STAT formation; D) MAPK pathway; induced phosphorylation of p38, JNK, and ERK, E) p53 pathway; induced phosphorylation of p53 and induction of apoptosis. (Adopted from Ref. 99 under the terms of the Creative Commons Attribution License (CC BY)).

5.5. NF-κB, p53 and apoptotic signaling

Quercetin triggers the stimulation of 5-fluorouracil-initiated apoptosis in a p53-dependent manner [100]. A similar study also observed that quercetin and p53 work in a synergistic manner [100]. In another study, quercetin along with doxorubicin down regulates the Bcl-xl in a p53-dependent phase [101]. Quercetin was found to promote cell death-associated gene expression including p53 along with downregulation of AKT and Bcl-2 expression [102]. It was also found that quercetin suppresses the mTOR expression simultaneously activation of p53, Sestin-2 via AMPK. In a relevant study performed using nano-quercetin of HepG2 cells it was observed that it activates the p53-ROS crosstalk and triggers apoptosis along with modification at the epigenetic level and cell cycle arrest at the sub-G phase [103]. Another study showed that quercetin activates the p21, p53 and GADD45 signalling mechanism along with simultaneous suppression of JNK mediated through Foxo3a [104].

6. Protective mechanism of EVs in HCC

Extracellular vesicles are known to enhance the anti-cancerous potential by hampering several signalling pathways involved in the metastasis of HCC. In one of the previously published studies, it was found that the Vps4A level is higher in EVs derived from HCC which inhibits the PI3K-Akt signalling pathway that in turn inhibits the HCC progression and metastasis [105]. EVs also served as a mediator in the regulation of intracellular micro-RNA (miRNAs). In one of the studies it was found that Vps4A exhibits two oncogenic miRNAs (miR-27b-3p and miR-92a-3p) and that is found to be upregulated in SMMC-Vps4A [105].

In addition to this SMMC-Vps4A also possesses miR-193a-3p, miR-320a, and miR-132-3p as tumour suppressor miRNAs [105]. Similar study further detected six tumor suppressor miRNAs

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(miR-122-5p, miR-33a-5p, miR-34a-5p, miR-193a-3p, miR-16-5p, and miR-29b-3p) that showed upregulated trend [105]. The findings of this study were supported by the other authors and they found the role of tumour suppressor miRNAs miR-122-5p, miR-33a-5p, miR-34a-5p, miR-16-5p, and miR-29b-3p in HCC [106]. The authors of a previously published study performed western blotting and found that overexpression of Vps4A leads to the inactivation of the PI3K/Akt signalling pathway that also modulates the miRNAs [105]. So, it was concluded that Vps4A showed a therapeutic target against HCC in miRNAs dependent and independent manner and can be explored as checkpoints for the treatment of patients with HCC.

Another study found that expression of *SENP3-EIF4A1* and *SENP3-EIF4A1* in secretory EVs suppress the HCC proliferation through miR-9-5p mediated action of *ZFP36* [107]. Moreover, IncRNA 85 controls the cancer cell invasion by acting on miR-324-5p by regulating the expression of *MMPs*, *ETS1* and *SP1* in HCC [108]. EVs containing miR-320a showed a protective effect against HCC through suppression of the PBX3/ERK1/2/CDK2 signaling pathway [109].

Mesenchymal stromal cells (MSCs) derived EVs are known to exhibit anti-cancer properties and can be explored for the treatment of HCC. A previous study showed that umbilical cord-derived MSCs significantly improved the anti-tumour response of NKT cells in liver cancer by inhibiting oxidative stress [110]. In another study, it was found that miR-122 provides an anti-cancer effect against HCC by suppressing the PI3-K/Akt signaling pathway as shown in Figure 6 [111]. In another study, it was found that fibroblast-derived EVs exhibit less quantity of miR-320a and thus inhibit HCC by suppressing the MAPK signalling pathway [109]. Another study claimed that fibroblast-derived EVs were rich in miR-150-3p and exhibit anti-cancer properties against HCC [112]. Some studies found that miR-195 presence in fibroblast-derived EVs suppresses the activation of VEGF, CDC42, CDK1, CDK4, CDK6, and CDC25 and is considered to be a new therapeutic target for HCC [113,114]. In another study, mi331-3p also inhibits the progression of HCC through the regulation of BAK1 [115].

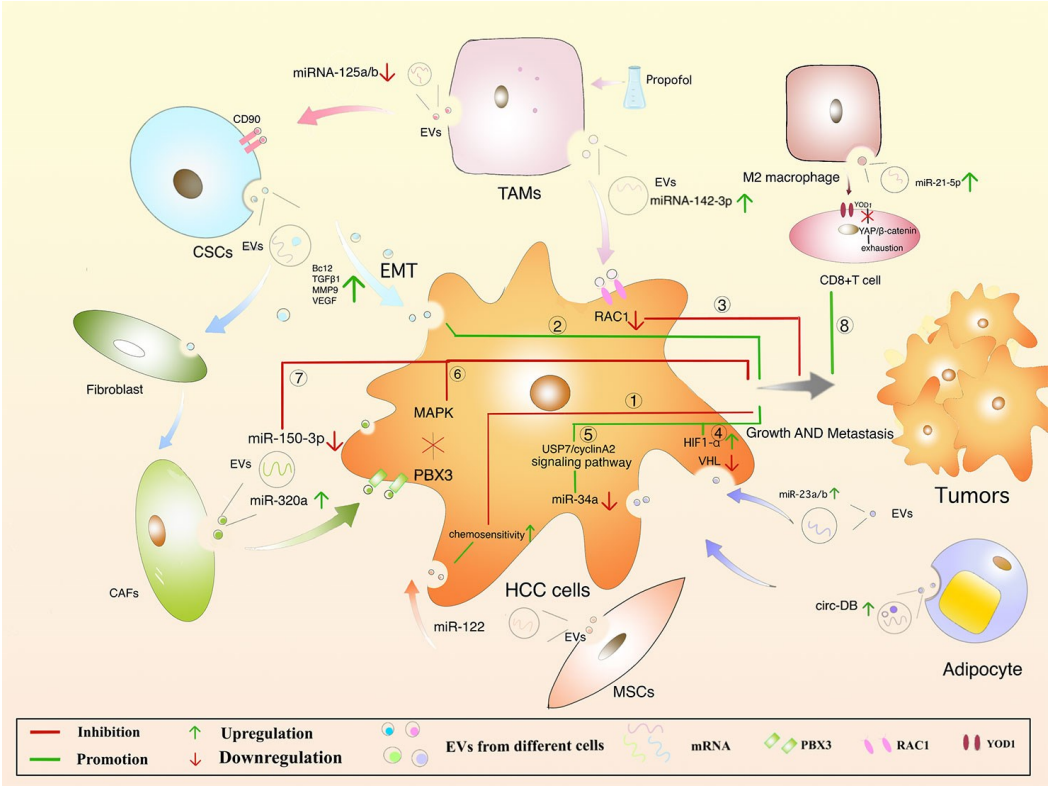

Figure 6. Regulation of hepatocellular carcinoma by different cell-derived EVs. (Adopted from Ref. 111 under the terms of the Creative Commons Attribution License (CC BY)). TAMs, tumor-associated macrophages; CSCs, cancer stem cells; CAFs, cancer-associated fibroblasts; EMT, epithelial-mesenchymal transition.

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7. Challenges and perspectives of anti-cancer EVs biopharmaceuticals

In the last decades, the unrelenting progress of biologics encourages to development of a thorough understanding and technological advancement of biopharmaceutical manufacturing procedures. This sharp evolution has deepened the interest of the biopharmaceutical industries in process analytical technology (PAT), which is known as a system for designing, analyzing and controlling the manufacturing of the products along with ensuring the final quality of the product [116]. Nanoparticles are well known to deliver many biologics including proteins, peptides and antibodies. However, such particles face severe challenges including physiological barriers, fast wash-off from targeted sites, poor permeation-retention effect etc. With the spent of time, technological advancements help researchers to overcome several hurdles with the advent of extracellular vesicles. EVs if compared to synthetic drug delivery nanomaterial exhibits natural site targeted features along with improved stability, biocompatibility, and increased bioavailability. Therefore, EVs are considered to be the biggest opportunity for the biopharmaceutical industry to be used as drug/nanoparticle delivery vehicles. Although substantial breakthroughs were fabricated using these engineered EVs as anti-cancer therapy still some challenges may hinder the path to making a bench to bedside products. The complex structure of EVs is associated with a high degree of inconsistency that might affect the therapeutic properties. Moreover, large-scale isolation and purification approaches of EVs still compromised their yield. Researchers nowadays concentrate their research on testing customized EVs in preclinical animal models but data is still lacking in clinical trials. Cargo loading efficiency is still an un-addressable issue and needs serious attention. Summing up the issues of biosafety, bioavailability, biocompatibility, and biostability are some of the peculiar challenges for future clinical translational research.

Conclusions

Quercetin is a polyphenolic flavonoid exhibiting anti-cancerous features that exert its therapeutic mechanism in hepatocellular carcinoma through dysregulation of several signalling mechanisms including PI3K/AKT, NF-κB, P53, Wnt/β-catenin, MAPK, JAK/STAT and Hedgehog pathway. Moreover, quercetin is known to modulate several intracellular signalling biologics including TNF-α, Bax, Bcl-2, caspases, and VEGF. The anticancer potential of quercetin was extensively studied in various cancers including hepatocellular carcinoma. However, the majority of the research was evident in preclinical studies. Studies are lacking in demonstrating clinical trials. EVs derived from mesenchymal lineages were considered to trigger anti-cancerous effects through several miRNAs and IncRNAs. Not a single study was conducted in past that demonstrated the synergistic effect of quercetin and mesenchymal stem cells derived EVs at the clinical trial phase. There are significantly high expectations of such phase III trials focusing on all stages of HCC.

Abbreviations

HCC: Hepatocellular carcinoma
UVR: Unrelenting virological response
NASH: Non-alcoholic steatohepatitis
NAFLD: non-alcoholic fatty liver disease
EVs: Extracellular vesicles
AAPC: Average annual percent change
HKLC: Hong Kong Liver Cancer
CLIP: Cancer of the liver Italian program
BCLC: Barcelona clinic liver cancer
AASLD: American Association for the Study of Liver Diseases

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