Dairy consumption and hepatocellular carcinoma risk

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Abstract: This review provides epidemiological and translational evidence for milk and dairy intake as critical risk factors in the pathogenesis of hepatocellular carcinoma (HCC). Large epidemiological studies in the United States and Europe identified total dairy, milk and butter intake with the exception of yogurt as independent risk factors of HCC. Enhanced activity of mechanistic target of rapamycin complex 1 (mTORC1) is a hallmark of HCC promoted by hepatitis B virus (HBV) and hepatitis C virus (HCV). mTORC1 is also activated by milk protein-induced synthesis of hepatic insulin-like growth factor 1 (IGF-1) and branched-chain amino acids (BCAAs), abundant constituents of milk proteins. Over the last decades, annual milk protein-derived BCAA intake increased 3 to 5 times in Western countries. In synergy with HBV- and HCV-induced secretion of hepatocyte-derived exosomes enriched in microRNA-21 (miR-21) and miR-155, exosomes of pasteurized milk as well deliver these oncogenic miRs to the human liver. Thus, milk exosomes operate in a comparable fashion to HBV- or HCV- induced exosomes. Milk-derived miRs synergistically enhance IGF-1-AKT-mTORC1 signaling and promote mTORC1-dependent translation, a meaningful mechanism during the postnatal growth phase, but a long-term adverse effect promoting the development of HCC. Both, dietary BCAA abundance combined with oncogenic milk exosome exposure persistently overstimulate hepatic mTORC1. Chronic alcohol consumption as well as type 2 diabetes mellitus (T2DM), two HCC-related conditions, increase BCAA plasma levels. In HCC, mTORC1 is further hyperactivated due to *RAB1* mutations as well as impaired hepatic BCAA catabolism, a metabolic hallmark of T2DM. The potential HCC-preventive effect of yogurt may be caused by lactobacilli-mediated degradation of BCAAs, inhibition of branched-chain α-ketoacid dehydrogenase kinase via production of intestinal medium-chain fatty acids as well as degradation of milk exosomes including their oncogenic miRs. A restriction of total animal protein intake realized by a vegetable-based diet is recommended for the prevention of HCC.

Keywords: Branched-chain amino acids (BCAAs); dairy products; hepatocellular carcinoma (HCC); exosomal microRNAs; mechanistic target of rapamycin complex 1 (mTORC1)

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Introduction

Hepatocellular carcinoma (HCC) mortality rates have increased over recent decades (1-4). Major risk factors for HCC are chronic liver disease and cirrhosis due to hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infection, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), steatohepatitis, intake of aflatoxin-contaminated food, type 2 diabetes mellitus (T2DM) and obesity (1-6). Although increased dairy product intake has been associated with increased risk of several cancers (7-11), epidemiological studies on dairy and milk consumption in HCC are sparse. Five older case-control studies with small patient numbers presented conflicting results (12-16). Recently, Yang *et al.* (17) assessed the associations of dairy products with the risk of HCC development among 51,418 men and 93,427 women in the Health Professionals Follow-Up Study and the Nurses' Health Study. After adjustment for known

HCC risk factors, higher total dairy intake was associated with an increased risk of HCC. In accordance with the association of milk consumption and T2DM (18-20), there was an inverse association between yogurt intake and HCC risk (17). The European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (477,206 participants) demonstrated a significant positive HCC risk association with total dairy product intake, milk and cheese, but not yogurt (21). The Guangzhou Biobank Cohort Study (18,214 participants) showed that moderate (250–750 mL/week) versus high milk consumption (>750 mL/week) significantly increased the mortality rate for all cancers and raised the mortality rate of liver cancers from 3.8 to 7.0 per 10,000 person-years, respectively (22).

In 2017, the per capita milk consumption was 65.2 L in the United States and 52.2 L in Germany, respectively (23,24). Since the 1950s, the annual consumption of cheese, a rich source of branched-chain amino acids (BCAAs), has increased four- to five-fold in Western societies (25,26).

It is the intention of this review to present molecular mechanisms that may explain dairy-induced hepatocancerogenesis. For this purpose, it is important to keep in mind that milk represents a postnatal signaling system evolutionarily restricted to the breastfeeding period of mammals for the promotion of cell proliferation and growth (27-29).

Insulin-like growth factor 1 (IGF-1)

Circulating IGF-1 concentrations have been associated with higher cancer risk (30,31). A cross-sectional EPIC analysis of 4,731 men and women demonstrated that each 1 standard deviation (SD) increment increase in total dairy and dairy protein was associated with an increase in IGF-1 concentrations of 2.5% and 2.4%, respectively (32). A meta-analysis reported that circulating IGF-1 increased by 13.8 ng/mL in the milk intervention group compared with the control group (33). A recent study in Germany showed that each 400 g increment in daily dairy intake in adults was associated with 16.8 ng/mL higher IGF-1 serum concentrations, whereas each daily 200 g increment in milk was associated with 10.0 ng/mL higher IGF-1 (34). Remarkably, whey protein intake preferentially increased serum insulin levels, whereas casein raised serum IGF-1 levels (35).

The liver is the primary organ releasing IGF-1 into the systemic circulation (36,37). Dietary amino acids (AAs) and insulin induce hepatic IGF-1 expression and secretion (38,39) (*Figure 1*). Increasing AA concentrations enhance hepatic expression of IGF-1, peroxisome proliferator-activated receptor-γ (PPARγ) and mechanistic target of rapamaycin (mTOR) (38). Low protein diets are associated with lower cancer incidence and mortality rates in humans. Protein restriction inhibits tumor growth via attenuation of IGF-1/mTORC1 signaling (40). Individuals aged 50–65 with a high protein intake had a four-fold increase in the risk of cancer death during a follow-up of 18 years (41). Post-initiation development of aflatoxin B1 (AFB1)-initiated preneoplastic foci in Fischer 344 rat liver could be prevented by decreasing casein intake (42). Dietary casein reduction from 22% to 6% markedly inhibited hepatic tumor formation in HBV-transfected mice (43). A low casein diet also suppressed HBV-induced liver injury (44).

IGF-1/IGF-1 receptor signaling plays a critical role in hepatocarcinogenesis (45-51), whereas blocking of IGF-1 receptor pathway is regarded as a treatment option in HCC (52-60). Increased systemic IGF-1 levels and enhanced hepatic IGF-1 signaling characterize the initiation stage of HCC, whereas overt HCC with compromised liver function and liver cirrhosis is associated with decreased hepatic IGF-1 synthesis and low serum IGF-1 (45,61-68), a marker for liver reserve capacity in HCC patients (69).

BCAAs

The nutrient-sensitive kinase mechanistic target of rapamycin complex 1 (mTORC1) is overactivated in many cancers including HCC (70-79). Not only insulin and IGF-1 activate mTORC1 but also AAs (80,81), preferentially the BCAAs leucine, isoleucine and valine (82-88). In comparison with other animal- and plant-derived protein-rich nutrient sources, milk proteins are highly enriched in BCAAs (89) (*Table 1*).

Solute carrier family 7 member 5 (SLC7A5) plays a key role for leucine uptake in cancer cells (91,92). Oncogenic MYC stimulates SLC7A5 expression promoting BCAA import for tumorigenesis (93). High SLC7A5 expression in HCC is related with tumor size, tumor stage and shortened survival time (94). SLC7A5 expression is associated with increased expression of SLC3A2 (95). Canine HCCs exhibit 28 times higher SLC7A5 expression than normal hepatocytes (96). SLC7A5 also plays a critical role in hepatic cancer stem cells (HCSCs) (97). Cellular uptake of glutamine via SLC1A5 and its subsequent efflux in the presence of BCAAs is the rate-limiting step that activates mTORC1 (98). The bidirectional transporter SLC7A5/

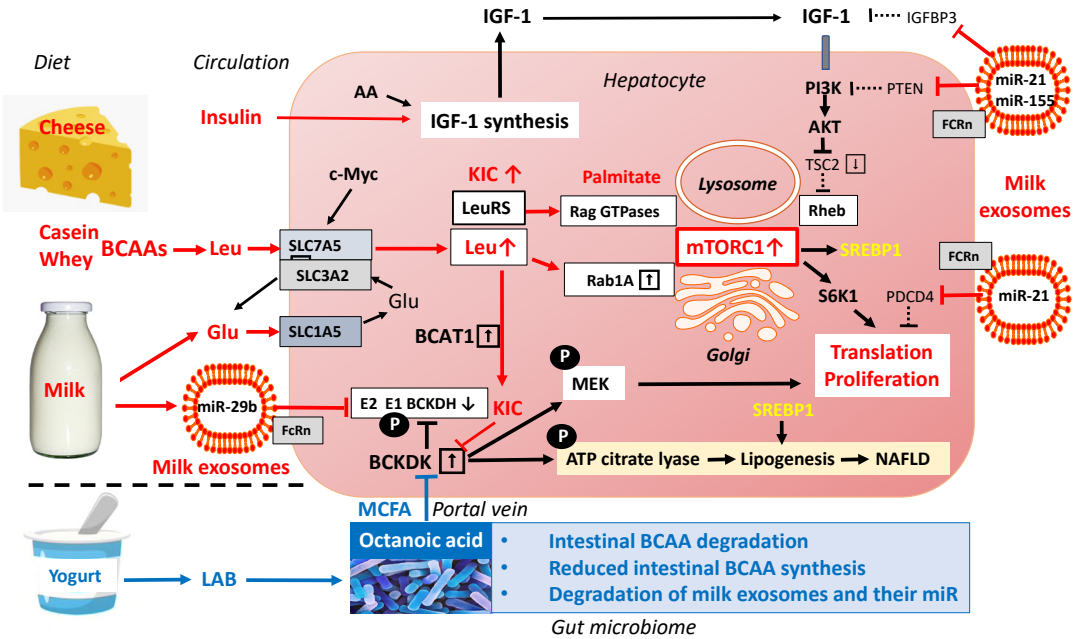

Figure 1 Working model illustrating potential molecular mechanisms of dairy products in the pathogenesis and prevention of hepatocellular carcinoma (HCC). Cheese is a rich source of branched-chain amino acids (BCAAs) and palmitate. Pasteurized milk provides BCAAs, glutamine, palmitate and bioavailable exosomal microRNAs (miR-21, miR-155, miR-29b). The amino acid transporter SLC7A5 is upregulated in HCC promoting leucine (Leu)-mediated activation of mechanistic target of rapamycin complex 1 (mTORC1). Branched-chain amino acid transaminase 1 (BCAT1) is upregulated in HCC, resulting in increased hepatic levels of α-ketoisocaproate (KIC), which in synergy with Leu activates mTORC1. Rab1A is also overexpressed in HCC promoting mTORC1 activation at the Golgi membrane. Activated mTORC1 enhances the expression of sterol regulatory element-binding protein 1 (SREBP1) and the kinase S6K1 promoting lipogenesis, translation and proliferation. Tuberin (TSC2) expression is decreased in HCC enhancing Rheb-mediated activation of mTORC1. Exosomal miR-21 and miR-155 inhibit the expression of insulin-like growth factor binding protein 3 (IGFBP3) and phosphatase and tensin homolog (PTEN) further augmenting phosphatidylinositol-3 kinase (PI3K)-AKT-mTORC1 signaling. MiR-21 suppresses programmed cell death 4 (PDCD4), a key suppressor of translation initiation. In HCC, the activity of branched-chain α-ketoacid dehydrogenase (BCKDH), the rate-limiting enzyme of BCAA catabolism, is decreased leading to increased hepatic levels of BCAA and KIC that activate mTORC1. In HCC, the activity of branched-chain α-ketoacid dehydrogenase kinase (BCKDK) is increased. BCKDK phosphorylates and thereby inactivates the E1 unit of BCKDH. Exosomal miR-29b inhibits the expression of the E2 core unit of BCKDH (dihydrolipoamide branched-chain transacylase), which is essential for the functional assembly of BCKDH. BCKDK also activates MEK and ATP citrate lyase increasing proliferation and lipogenesis. In contrast, yogurt-derived lactobacilli (LAB) increase intestinal levels of medium chain fatty acid (MCFA) octanoic acid, which reaches the liver directly via the portal vein and functions as an allosteric inhibitor of BCKDK thereby increasing BCAA catabolism. Further HCC-preventive effects of LAB are modifications of the gut microbiome and its metabolome with reduction of bacterial BCAA synthesis, degradation of milk exosomes and their oncogenic miRs.

Table 1 BCAA composition of milk proteins compared to animal meat and plant protein sources

Amino acid	g amino acids/100 g protein										
	Milk	Casein	Whey	Codfish	Chicken	Egg	Beef	Pork	Lentil	Bean	Soy
Leucine	10.4	10.4	11.1	1.69	1.83	1.00	2.16	1.72	2.11	2.02	2.84
Isoleucine	6.4	5.7	6.8	0.99	1.34	0.74	1.33	1.12	1.19	1.10	1.78
Valine	6.8	6.8	6.8	1.09	1.25	0.89	1.45	1.27	1.39	1.24	1.76

Table modified according to (89,90). BCAA, branched-chain amino acids.

SLC3A2 regulates the simultaneous efflux of glutamine and concomitant influx of leucine into cells (*Figure 1*) (98). Glutamine abundance thus improves leucine uptake and BCAA-mediated mTORC1 activation, a potential explanation for the high glutamine content of milk proteins (90). The leucine and glutamine content is increased in HCC tissue and is higher in high-grade compared with low-grade HCCs (99). HCC is thus a leucine addict like prostate cancer (100,101), another common milk-induced cancer of Western societies (7-9,11).

Chronic activation of mTORC1 is sufficient to cause HCC in mice and represents the key molecular link between HCC risk and dietary factors (71). Long-term cow's milk consumption in young mice increased liver BCAA levels and activated mTORC1-S6K1 (102). Hepatic mTORC1 promotes the expression of lipogenic genes and leads to the development of HCC (72,79). mTORC1 upregulates sterol regulatory element-binding protein 1 (SREBP1), which contributes to NAFLD (79).

In HCC tissues, the expression level of branched-chain amino acid transaminase 1 (BCAT1), which catalyzes the production of branched-chain α-ketoacids (BCKAs) from BCAAs, is significantly increased compared with adjacent tissues (103). Patients with high BCAT1 expression possess a lower overall survival than those with low BCAT1 expression (103). Leucine and its conversion product α-ketoisocaproate (KIC) are sensed by leucyl-tRNA synthase (LeuRS) (*Figure 1*) (104). LeuRS acts as a GTPase-activating protein (GAP) for RagD GTPase activating mTORC1 (105). In high fat-diet (HFD)-treated diet-induced obesity (DIO) mice, BCAA supplementation increased hepatic BCAA and BCKA levels and induced severe hepatic insulin resistance (IR) (104).

Rag small GTPases are critical mediators of AA-mTORC1 assembly and mTORC1 activation at the lysosomal compartment (106,107). AAs are also capable of activating mTORC1 in the absence of Rag GTPases dependent on Rab1A (108-110). In colorectal cancer (CRC), AAs stimulate Rab1A interaction with mTORC1, whereby Rab1A promotes the formation and activation of the Rheb-mTORC1 complex at the Golgi (110). In accordance with CRC, overexpressed Rab1A in HCC has been identified as a crucial driver for AA-mediated mTORC1 activation in hepatocancerogenesis (*Figure 1*) (111).

The activity of BCAA catabolic enzymes is suppressed in HCC resulting in increased BCAA levels activating mTORC1 (112,113). Either downregulation of expression and/or changes in post-translational modifications, e.g., hyperphosphorylation of BCKA dehydrogenase (BCKDH) by BCKDH kinase (BCKDK) impairs BCAA catabolism leading to BCAA accumulation with chronic activation of mTORC1 in HCC (112,113). In accordance with HCC, upregulated BCKDK has also been observed in CRC (114). Hepatic overexpression of BCKDK increased the activity of ATP-citrate lyase activating *de novo* lipogenesis (115) linking disturbed hepatic BCAA catabolism to steatosis hepatis and HCC.

HBV and HCV

Hepatic overexpression of HBV X protein (HBx) activates AKT and mTORC1 signaling (116,117) and induces the expression of α-fetoprotein (AFP), which attenuates the function of phosphatase and tensin homolog (PTEN) leading to increased AKT-mTORC1 signaling in HBx-transfected human liver cells and HCC (118). This AFP-mediated mechanism also promotes the initiation of HCC progenitor/stem cells (119). In addition, HBx induces microRNA (miR)-181a further targeting PTEN (120).

HCV activates mTORC1 via upregulation of the SLC7A5/SLC3A2 complex augmenting cellular leucine influx (121). Thus, persistent milk consumption synergizes with HBV-/HCV-overstimulated hepatic mTORC1 signaling.

IR and obesity

Aberrant liver metabolism promotes IR, a hallmark of NAFLD and T2DM. NAFLD patients with hepatic IR generally share a high risk of impaired fasting glucose associated with early T2DM. Many patients with T2DM experience NAFLD, non-alcoholic steatohepatitis (NASH), and severe complications such as cirrhosis and HCC (122). Notably, high intake of milk, but not meat, increased serum insulin levels and IR in 8-year-old boys (123). Positive associations between full-fat dairy, non-fermented dairy products and milk with pre-diabetes and newly diagnosed T2DM has been reported in the prospective Dutch Lifeline Cohort Study including 112,086 adults (19). There is a confirmed association between elevated BCAA plasma levels and IR (25,124-133). A genetic link between obesity-associated IR and impaired BCAA catabolic gene expression in human and mouse models has been reported (134). In genetically obese (ob/ob) mice, the deficiency of BCKDH resulted in an accumulation of BCAAs and BCKAs, which both activate mTORC1 (134). Restoring BCAA catabolic

flux via inhibition of BCKDK reduced BCAA and BCKA abundance and markedly attenuated IR in ob/ob mice. Similar outcomes were achieved by reducing protein intake, whereas increasing BCAA intake did the opposite. Thus, compromised BCAA catabolism is a common hallmark of IR, T2DM and HCC (112,113,134), metabolic deviations overactivating mTORC1 (18,89). In mice, BCAA tissue levels were directly related to liver tumor development and tumor size associated with dietary BCAA intake and mTORC1 activity (112).

Single nucleotide polymorphisms of fat mass and obesity-associated gene (FTO) are related to body mass index and obesity. FTO is a N^6-methyladenosine (m^6A) demethylase that acts as the most common mRNA modification in normal and tumor cells linking obesity to cancer (135). In murine and human cell lines, total AA deprivation reduced FTO expression (136). FTO expression is regulated by essential AAs and couples AA levels to mTORC1 signaling through a demethylation-dependent mechanism. FTO is thus an AA sensor promoting growth and translation (137,138). It has been hypothesized that milk functions as an epigenetic amplifier of FTO-mediated transcription (139). Remarkably, FTO levels are upregulated in HCC tissue and correlate to a poorer prognosis, whereas FTO knockdown suppresses proliferation and *in vivo* tumor growth (140). FTO triggers the demethylation of pyruvate kinase 2 (PKM2) mRNA accelerating its translation. Overexpression of PKM2 activates mTORC1 signaling through phosphorylation-mediated inactivation of the mTORC1 inhibitor AKT1 substrate 1 (AKT1S1) (141). In addition, m^6A methylation of the 3' ε-stem loop results in destabilization of HBV transcripts, suggesting that m^6A has regulatory function for HBV RNA (142). Milk-mediated activation of FTO may thus accelerate HBV RNA transcription.

Milk fat

During late-stage NAFLD, fibrotic and cancerous cells can proliferate. HCC cells and normal hepatocytes are exposed to high concentrations of fatty acids from both surrounding tissue and circulating lipid sources. The saturated fatty acid palmitic acid (PA) exerts lipotoxic effects in activated human hepatic stellate cells (HSCs) and epithelial hepatoma cells (143). An HFD rich in PA is associated with lower insulin sensitivity (144). The National Institutes of Health-AARP Diet and Health Study confirmed an association between saturated fat intake and HCC (145). However, total amounts of triacylglycerols stored in hepatocytes are not the major determinant of lipotoxicity, whereas specific lipid classes in particular PA, cholesterol, lysophosphatidylcholine and ceramides damage liver cells (146,147).

Fatty acid transport protein 4 (FATP4) is a minor FATP in the liver. PA activation of FATP4 triggers hepatocellular apoptosis via altered phospholipid composition and steatosis by acylation into complex lipids (148). In HFD-treated DIO mice, BCAA supplementation increased plasma free fatty acid levels (104). O-GlcNAc transferase (OGT), which upregulates PA synthesis, is involved in metabolic reprogramming and IR and plays a key role in NAFLD-associated HCC (149).

Bovine milk contains 3.5% to 5% total lipid secreted by mammary gland epithelial cells as milk fat globules (MFGs). About 98% of milk lipids are triacylglycerols. PA is the predominant fatty acid of MFG triacylglycerols (150,151), which are hydrolyzed in the intestine and transported via chylomicrons into the systemic circulation. Upregulated PA absorption by activation of its hepatic transporters is evident in NASH (152). Notably, PA activates mTORC1 by enhancing its recruitment onto lysosomal membranes (153-155). Thus, milk fat-derived dietary PA may contribute to hepatic mTORC1 activation, which is involved in the pathogenesis of NASH (156-158).

Exosomal MFG-EGF factor 8 (MFG-E8) and TGF-β

Milk contains extracellular vesicles (EVs) that modulate numerous biological processes (159-161). EV subsets of milk can be separated by ultracentrifugation (161). The 100,000 ×g fraction contains the milk exosomes (50–100 nm) (162). Milk EVs including exosomes deliver RNAs, miRs and proteins protected by a lipid bilayer membrane, which confers resistance against their intestinal degradation (163).

Milk exosomes are bioavailable and distribute their cargo across species boundaries (164). They accumulate in liver following suckling, oral gavage and intravenous administration to mice and pigs (164). MFG-E8 is a major component of MFGs and milk exosomes (165-167). MFG-E8 (also called lactadherin) promotes proliferation of hepatocytes through the phosphatidylinositol-3 kinase (PI3K)/AKT/mTORC1 pathway and is a key regulator of cancer cell invasion, migration, and proliferation (168,169). MFG-E8 expression is higher in HCC cells compared with normal liver tissue (170). Overexpressed MFG-E8 promotes proliferation and migration of HCC cells, whereas MFG-

Table 2 Oncogenic effects of miR-21 in hepatocellular carcinoma

MiR-21 targets	Biological effects
IGFBP3	Increased IGF-1 signaling
PTEN	Increased PI3K-AKT-mTORC1 signaling
PDCD4	Increased translation
HEPN1	Reduced apoptosis
NAV3	Increased proliferation
RECK	Matrix metalloproteinase activation and metastasis
TIMP3	Matrix metalloproteinase activation and metastasis
IL-12p35	Reduced activation of natural killer cells and cytotoxic T lymphocytes, reduced anti-tumor immune defense
KLF5	Promotion of HCC cell migration and HCC progression

IGF-1, insulin-like growth factor-1; IGFBP3, IGF binding protein 3; PTEN, phosphatase and tensin homolog; PI3K, phosphatidylinositol-3 kinase; mTORC1, mechanistic target of rapamycin complex 1; PDCD4, programmed cell death 4; NAV3, navigator 3; RECK, reversion-inducing cysteine-rich protein with Kazal motifs; TIMP3, tissue inhibitor of metalloproteinase 3; KLF5, Kruppel-like factor 5; HCC, hepatocellular carcinoma.

E8-neutralizing antibodies inhibited HCC cell proliferation and migration (170).

The development of HCC is also associated with alterations in transforming growth factor-β (TGF-β) signaling (171-175). Increased numbers of TGF-β^+ regulatory T-cells (Tregs) have been detected in the peripheral blood of HCC patients (174). Ki-67 expression is also associated with TGF-β1 and deteriorates the prognosis of patients with HBV-related HCC (175). The Th17/Treg ratio is an independent factor influencing the occurrence of HCC in HBV-infected patients (176).

Milk exosomes transport TGF-β to the milk recipient (177). In mice, bovine milk exosomes promoted Th17 differentiation, which could be suppressed by anti-TGF-β antibodies (177). Thus, milk exosomes via MFG-E8 may stimulate hepatocyte proliferation and via TGF-β impair immunological surveillance.

Exosomal miRs

Exosomes and their miR cargo play an important role in HCC development and treatment (178-186). HCC-derived exosomal miR-21 contributes to tumor progression by converting HSCs to cancer-associated fibroblasts (CAFs) (187). HBx upregulates miR-21, which downregulates programmed cell death 4 (PDCD4) in HCC (188). HCC cell apoptosis was suppressed through HBx-induced miR-21 by targeting interleukin 12 (IL-12) (189,190). MiR-21 compromises IL-12-mediated anti-cancer activity including activation of natural killer (NK) cells and cytotoxic T lymphocytes (191). The secretion of miR-21-enriched exosomes by HBV-infected hepatocytes is thus a potential viral escape mechanism from host innate immune responses (192,193).

It is conceivable that milk-derived exosomes and their miR cargo modify hepatocyte transcriptional activity. After oral or retroorbital administration, bovine milk exosomes accumulated in the liver of mice (164,194) and apparently transfer their miRs into hepatocytes (195). Part of bovine milk exosome uptake is mediated by bovine immunoglobulin G (IgG), which binds to human neonatal Fc receptor (FcRn) (196) that is highly expressed in adult human liver (197).

MiR-21

MiR-21 is a well-established oncomiR whose steady-state tissue levels are commonly increased in many malignancies including HCC (198-206). MiR-21 promotes migration and invasion by the miR-21-PDCD4-AP-1 feedback loop in human HCC (198). MiR-21 activates mTORC1 and mTORC1-dependent translation via targeting PTEN and PDCD4 (207-209). Cancer susceptibility gene HEPN1 is frequently silenced in HCC, whereas exogenous HEPN1 exhibits antiproliferative effect on HepG2 cells, suggesting that silencing of HEPN1 may be associated with hepatocancerogenesis (210). MiR-21 promotes cell proliferation and migration in human HCC by targeting HEPN1 and Navigator 3 (NAV3) (199,211) (*Table 2*).

Circulating miR-21 serves as a biomarker for HCC and correlates with distant metastasis (199,211-214). The 5-year overall survival of a high miR-21 expression group was significantly shorter than that of a group with low miR-21 expression (215,216). Furthermore, overexpression of miR-21 is associated with HCC recurrence in patients with HBV-mediated HCC undergoing liver transplantation (217). High-mobility group box 1 (HMGB1) induces miR-21 in HCC repressing matrix metalloproteinase (MMP) inhibitors reversion-inducing cysteine-rich protein with Kazal motifs (RECK) and tissue inhibitor of metalloproteinase 3 (TIMP3), which regulate HCC

progression and metastases (218). Thus, upregulated miR-21 promotes various steps of hepatocancerogenesis (219-224). In contrast, degradation of miR-21 or treatment with anti-miR-21 suppressed HCC growth, induced apoptosis and reduced resistance to sorafenib and cisplatin (225-228).

Importantly, miR-21 is a signature miR of commercial milk (229,230) and a consistent component of milk exosomes (230). In human volunteers, increased plasma miR-21 levels have been reported 6 hours after milk consumption (231). Because human and bovine miR-21 sequences are identical (232), milk-derived exosomal miR-21 and HCC-induced exosomal miR-21 may synergistically promote hepatocancerogenesis (219-223,233).

Commercial milk obtained from persistently pregnant cows contains androgenic hormones (234,235). Mean milk androstenedione concentrations of pregnant cows are three-fold higher compared to non-pregnant cows (236,237). High affinity binding sites of androgens have been characterized in primary HCC cells (238). Androgen receptor activation by androst-5-ene-3,17-dione, androst-5-ene-3β,17β-diol, dihydrotestosterone, and 5α-androstane-3β increased miR-21 transcription in HepG2 cells (239). Thus, milk-derived androgenic steroids may enhance oncogenic miR-21 expression.

MiR-155

HBV and HCV are involved in HCC pathogenesis and affect the expression of miRs (240). MiR-155 is an important oncomiR driving HCC (241-243). The expression of miR-155 is upregulated in tissues and serum of patients with HCC (241-244). HBV via suppression of zinc finger and homeodomain protein 2 (ZHX2), also known as α-fetoprotein regulator 1, promotes proliferation of HCC through miR-155 activation (245). Overexpressed miR-155 in HCV-induced HCC activates Wnt signaling (246,247). Epstein-Barr virus (EBV) infection, also observed in HCC (248), as well increased the expression of miR-155 (249,250).

Both miR-21 and miR-155 directly target PTEN and thereby activate AKT-mTORC1 signaling (251,252). HCC exosome-mediated transfer of miR-155 contributes to HCC cell proliferation by targeting PTEN (252). Other targets of miR-155 in HCC are forkhead box O3A (FoxO3a) (253,254), collagen triple helix repeat containing 1 (CTHRC1) (255), AT-rich interactive domain 2 (ARID2) (256), sex-determining region Y box 6 (SOX6) (257), F-box and WD40 domain protein 7 (FBXW7) (258), and Kruppel-like factor 5 (KLF5) (259).

FoxO3a exerts antitumor properties in HCC, inducing the expression of proapoptotic genes, or interfering with signaling cascades commonly altered in HCC such as Wnt/β-catenin, PI3K/AKT/mTORC1 or MAPKs pathways (260). MiR-155 overexpression increases metastasis- and antiapoptosis-related protein expression and decreases proapoptosis-related protein expression (255,256). As shown in an HCC cell line, ARID2 knockout disrupts DNA repair processes resulting in susceptibility to carcinogens and potential hypermutation (261). Decreased expression of SOX6, which plays critical roles in cell fate determination, differentiation and proliferation, confers a poor prognosis in HCC (262). Because FBXW7 mediates ubiquitination-dependent degradation of c-MYC and mTOR (263,264), miR-155-mediated suppression of FBXW7 is likely to affect HCC tumorigenesis. MiR-155 in synergy with miR-21 suppresses IGF binding protein 3 (IGFBP3) activating IGF/IGFR signaling in HCC (265,266) (*Figure 1*). TGF-β1, a component of milk exosomes (177), promotes the expression of miR-155. Increased levels of miR-155 in HCC cells accelerate epithelial-mesenchymal transition (EMT), activate PI3K-serum/glucocorticoid-regulated kinase 3 (SGK3)-β-catenin signaling, promote cellular invasion and migration (267,268). Notably, TGF-β1 promotes the expression miR-155 in HCC (267). MiR-155 is also upregulated in diffuse large B-cell lymphoma (269,270), another malignancy related to milk consumption (10). A relationship between serum miR-155 and telomerase expression has been observed in HCC (271). MiR-155 downregulates suppressor of cytokine signaling 1 (SOCS1), resulting in activation of STAT3 and STAT3-induced transcription of miR-21 (272,273). MiR-155 targets tumor protein p53-inducible nuclear protein 1 (TP53INP1) regulating liver CSC acquisition and self-renewal (274). Under hypoxic conditions, HCC cells secrete miR-155-enriched exosomes enhancing angiogenesis in endothelial cells (275). Finally, miR-155 has been identified as a biomarker for tumor recurrence and survival of HCC patients following orthotopic liver transplantation (276) (*Table 3*).

The role of EVs in mediating HCC progression and metastasis as well as HCC therapy is in the focus of recent research (178,277). Importantly, miR-155 is enriched in exosomes released from HCC cells and exosome-derived miR-155 is transferred into targeted cells increasing HCC cell proliferation (251,252). HCV proteins associate with the membrane tetraspanin CD81 for HCV infection of

Table 3 Oncogenic effects of miR-155 in hepatocellular carcinoma

MiR-155 targets	Biological effects
IGFBP3	Increased IGF-1 signaling
PTEN	Increased PI3K-AKT-mTORC1 signaling
SOCS1	Enhanced STAT3 signaling and miR-21 expression
TP53INP1	Liver cancer stem cell acquisition and self-renewal
FOXO3A	Reduced pro-apoptotic signaling
CTHRC1	Disturbed WNT/β-catenin signaling
ARID2	Decreased DNA repair
SOX6	Disturbed differentiation and increased proliferation
FBXW7	Increased c-MYC and mTOR expression

IGF-1, insulin-like growth factor-1; IGFBP3, IGF binding protein 3; PTEN, phosphatase and tensin homolog; PI3K, phosphatidylinositol-3 kinase; mTORC1, mechanistic target of rapamycin complex 1; SOCS1, suppressor of cytokine signaling 1; STAT3, signal transducer and activator of transcription 3; TP53INP1, p53-inducible nuclear protein 1; CTHRC1, collagen triple helix repeat containing 1; ARID2, AT-rich interactive domain 2; SOX6, sex-determining region Y box 6; FBXW7, F-box and WD40 domain protein 7.

hepatocytes (278). HCV envelope glycoprotein E2 binds to CD81 associated with exosomes that were captured from the plasma of HCV-infected patients (279). In analogy to HCV, HBx can tweak the exosome biogenesis machinery both by enhancing neutral sphingomyelinase 2 activity as well as by interacting with exosomal neutral sphingomyelinase 2, CD9 and CD81 (280). Enhanced secretion of exosomes by HBx-expressing cells has been confirmed with co-localization of HBx with CD9 and CD63 (280).

Milk exosomes also contain CD9, CD63 and CD81, all dominant milk exosome membrane proteins (165,166) that may augment HBV and HCV transmission. In analogy with HBV- and HCV-induced exosome secretion, milk exosome-derived miR-21 and miR-155 (229,230,281-284) via milk exosome uptake may modify hepatocyte gene transcription promoting HCC.

Notably, the liver is the major organ for the uptake of orally administered milk exosomes (164,284). Thus, not only HCC-derived exosomal miR-155 but also milk exosome-derived miR-155 may converge in exosome-induced hepatocancerogenesis (285). Of interest, the combined panel of miR-155, miR-96, miR-99a and AFP has a higher sensitivity and specificity for the diagnosis of HCC when compared with each single marker (244).

MiR-29b

MiR-29b is a bioavailable bovine milk miR, which increases in plasma and peripheral blood mononuclear cells of healthy volunteers 4 to 6 hours after milk consumption (195). MiR-29b plays a key role in hepatic IR (18,89) and is related to impaired hepatic BCAA catabolism (286-288). Human and bovine miR-29b exhibit identical sequences (mirbase.org). Intriguingly, miR-29b inhibits BCAA catabolism via targeting the E2 core component (DBT) of BCKDH (*Figure 1*) (289). In this regard, milk-derived miR-29b may augment compromised BCAA catabolism in HCC (112,113). Milk miR-29b-mediated suppression of BCAA catabolism during the breastfeeding period might conserve BCAAs for mTORC1-dependent growth and BCAA incorporation into structural and functional proteins such as albumin (290).

Milk exosomes: potential carriers of oncogenic viruses

Host exosome pathways are hijacked by viruses such as HBV, HCV, and EBV (291-297). Circulating virus particles may interact with exosomal tetraspanins of milk (165,166), a potential mechanism promoting cellular virus uptake. The detection of a novel HCV-like virus (BovHepV) in domestic cows with liver tropism is of concern (298,299), because it is not yet known whether BovHepV is able to enter milk exosomes and modify hepatic gene expression of milk consumers.

Furthermore, virus-like small circular single-stranded DNA (ssDNA) molecules have been isolated from commercial milk (300-302). These replication-competent genomic DNA sequences called bovine meat and milk factors (BMMF1 and BMMF2) are regarded as a specific class of infectious agents spanning between bacterial plasmid and circular ssDNA viruses with similarities to the genome structure and replication of hepatitis deltavirus (303). The possibility that these potentially oncogenic DNA sequences are transported via milk exosomes is under investigation (Harald zur Hausen, personal communication).

Milk exosomes for drug delivery

There are recent efforts to use exosomes for drug transfer as anti-cancer agents (277,304-307) and HCC treatment

Table 4 Effects of hepatocancerogenic agents on BCAA metabolism

Exposures	Effects
Hepatitis C virus	Upregulation of SLC3A2/SLC7A5 increasing cellular leucine influx
Alcohol	Increased plasma BCAA levels
Aflatoxin M1	Increased hepatic BCAA levels
Obesity	Increased plasma BCAA levels
Insulin resistance and type 2 diabetes	Increased BCAA plasma levels and decreased BCAA catabolism
Milk protein intake	Increase of hepatic BCAA levels and activation of mTORC1
Milk exosomal miR-29b	Inhibition of DBT decreasing BCKDH-mediated BCAA catabolism increasing cellular BCAA levels activating mTORC1

BCAA, branched-chain amino acid; SLC3A2, solute carrier family 3, member 2 (4F2hc); SLC7A5, solute carrier family 7, member 5 (LAT1); BCKDH: branched-chain α-ketoacid dehydrogenase complex; mTORC1, mechanistic target of rapamycin complex 1; DBT, dihydrolipoamide branched-chain transacylase.

(180,308,309). Some investigators promote cow milk-derived exosomes as potential carriers for drug delivery (196,284,310,311). However, it should be kept in mind that milk exosomes *per se* carry oncogenic miRs (miR-21, miR-155) and TGF-β that should not augment oncogenic signaling cascades (160).

Aflatoxin

Ruminants metabolize AFB1 ingested by contaminated food to aflatoxin M1 (AFM1), the hydroxylated mycotoxin, which is excreted into milk (312,313). There is concern about an increase of AFM1 concentrations in milk of maize-fed cows due to the climate change (314). The International Agency for Research on Cancer classified AFB1 and AFM1 as human carcinogens of group 1 (315-317). AFM1 is relatively stable during pasteurization, storage and processing (318-320). More than 50% of HCC patients from high aflatoxin exposure areas harbor a codon 249 G to T transversion in the p53 tumor suppressor gene, which is found to be consistent with the mutagenic specificity of AFB1 observed *in vitro* (321). This is of concern as milk signaling *per se* attenuates p53 activity by various mechanisms including AKT-mediated activation of mouse double minute 2 (MDM2) (322,323). As demonstrated with ^{1}H NMR spectroscopy, AFB1 exposure of rats elevated hepatic BCAA levels (324). In accordance, AFM1 exposure of HepG2 cells enhanced cellular BCAA levels (325). Consumption of alcohol (2,000 kcal added to the diet) in human alcoholics for 2 to 4 weeks as well increased BCAA levels (326). In baboons, chronic alcohol intake over years increased plasma leucine concentrations and enhanced plasma BCAA levels with steatosis hepatis as well as hepatitis-fibrosis (*Table 4*) (326).

Liver cirrhosis and advanced HCC

In contrast to the unnoticed induction phase of BCAA-mTORC1-driven hepatocancerogenesis, direct supplementation of BCAAs in patients with advanced liver cirrhosis may reduce profibrotic signaling and prevent progressive liver failure (327). In patients with overt HCC, BCAA supplementation improved overall and disease-specific survival in those patients with low BCAA to tyrosine ratios despite having normal albumin levels (328). Overexpression of platelet-derived growth factor C (PDGF-C) promotes liver fibrosis, which is preceded by activation and proliferation of HSCs (329). In PDGF-C transgenic mice, an anti-fibrotic effect of BCAAs has been observed (330). In HSCs, BCAAs restored TGF-β1-stimulated expression of profibrotic genes, whereas in hepatocytes, BCAAs restored TGF-β1-induced apoptosis, lipogenesis, and Wnt/β-catenin signaling, and inhibited the transformation of WB-F344 rat liver epithelial stem-like cells. The inhibitory effect of BCAA on TGF-β1 signaling was mTORC1-dependent, suggesting a negative feedback regulation from mTORC1 to TGF-β1 (330). TGF-β1 activates HSC proliferation and primes HSCs for extracellular matrix deposition and scar contraction (331). In HFD-treated mice, HCC induction by the hepatic carcinogen diethylnitrosamine (DEN) resulted in mTORC1 inhibition, increase of IL-6, activation of STAT3 and HCC development (332). In a rat model of DEN-induced liver fibrosis and HCC, a diet containing either 3% casein, 3% or 6% BCAAs for 13 weeks beginning 6 weeks after DEN administration demonstrated less dysplastic foci and less numbers of HCC in the BCAA groups at 16 weeks of DEN administration compared to the casein group (333). However, at 19 weeks of DEN, there was a trend to higher HCC numbers in the BCAA groups compared to casein controls (333). In contrast, attenuation of mTORC1 activity by low-dose oral rapamycin reduced fibrogenesis, improved liver function, and prolonged survival in rats with bile duct ligation-induced liver cirrhosis (334). Obviously, the type

of model, target cells and stage of HCC development are of importance for HCC-promoting or HCC-preventing effects of BCAAs.

Nevertheless, BCAA-mediated activation of hepatic mTORC1 is generally accepted to promote HCC development, progression and spreading (78,79,335,336). Activation of mTOR is more intense in the tumor edge, reinforcing its role in HCC proliferation and spreading (336). In addition, mTOR is overexpressed in multinodular HCC and is associated with increased post-liver transplantation tumor recurrence rates (336). mTORC1 upregulates SREBP1 via crosstalk with the STAT5 pathway, which contributes to NAFLD-related HCC pathogenesis (79). In HBV-associated tumorigenesis, the mTORC1 signal cascade also plays an important role in promoting *de novo* lipogenesis through activation of SREBP1 and ATP-citrate lyase (337). Loss-of-function of tuberin (TSC2), a key negative regulator of mTORC1, is common in HCC (338). Finally, the majority of therapeutic interventions in HCC aim at decreasing AKT-mTORC1 signaling (339-342).

Inverse relation between yogurt intake and HCC risk

Epidemiological studies confirmed an inverse relationship between yogurt consumption and the risk of HCC (17,21) as well as T2DM (20,343,344), two related pathologies exhibiting disturbed BCAA catabolism (112,113,132-134). The combination of *Streptococcus thermophiles* and *Lactobacillus delbrueckii subsp. bulgaricus* is the classic starter in yogurt production. Lactic acid bacteria (LAB) degrade milk's natural signaling capacity. LAB modify intestinal and systemic BCAA homeostasis, degrade milk proteins, and produce a-ketoacids including KIC (345-347), a physiological inhibitor of BCKDK (348). Probiotic yogurts also contain the strong BCKDK inhibitor octanoic acid (349,350). Via the portal vein, octanoic acid is taken up by the liver (*Figure 1*) (351). In rats, oral administration of octanoic acid activated hepatic BCKDH via suppression of BCKDK activity reducing BCAA plasma concentrations (350). *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* increase the intestinal ratio of *Bacteroidetes* to *Firmicutes* associated with increased butyrate production (352-354). The butyrate-derivative phenylbutyrate (PB) is an allosteric inhibitor of BCKDK and reduces BCAA plasma concentrations (355). Activated BCKDK promotes CRC via direct MEK phosphorylation (356). A cancer-preventive effect of PB has been observed in two liver carcinoma cell lines (Bel-7402, HepG2) (357-360). Furthermore, LAB-mediated fermentation of milk decreased the size of milk exosomes, exosome protein and miR-21 as well as miR-29b content (361). Yogurt intake increased the density of LAB and *C. perfringens* and reduced Bacteroides (362). *Bacteroides vulgatus* is a major producer of intestinal BCAAs that has been related to IR (363).

Thus, the HCC- and T2DM-preventive effect of yogurt consumption may reside in LAB-mediated improvements of intestinal and systemic BCAA homeostasis via suppression of intestinal BCAA synthesis and hepatic BCKDK resulting in upregulated BCKDH activity and reduced plasma BCAA levels (*Figure 1*).

Summary and conclusions

On the basis of epidemiological and translational evidence, it can be concluded that total dairy, milk and butter are critical risk factors of HCC. Increased intake of milk protein-derived BCAAs and saturated milk fat promote hepatic IGF-1 and mTORC1 activation. HCC-related deviations on BCAA catabolism combined with extensive BCAA intake promote excessive mTORC1 activation. Milk-derived exosomes and their oncogenic miRs apparently synergize with oncogenic signaling of HCC-derived exosomes in hepatocancerogenesis.

For HCC prevention, dietary reduction of BCAAs should be pursued in accordance with recommendations for the prevention of IR and T2DM (25,364). Notably, the most common antidiabetic drug metformin exhibits potential for the prevention and adjuvant treatment of HCC (365-368). Metformin inhibits mTORC1 (369,370), suppresses BCAT2 associated with a reduction of KIC (371), and reduces circulating BCAA levels (372), thus operates in the opposite direction of milk signaling (28,373).

The Canadian Dietary Guidelines recommend an animal protein-reduced and vegetable-accentuated diet (374), which apparently decreases the risk of HCC (375,376) including milk-related overall and cancer mortality (8,22,377-380). Milk exosomes and their oncogenic miRs have to be removed from the human food chain (160) as substantial evidence implies that milk exosome-derived oncogenic miRs promote HCC in synergy with HBV-/HCV-induced hepatic exosomes. Thus, accumulating evidence adds milk, cheese and butter with the exception of yogurt to the list of dairy-related risk factors of HCC (*Table 5*).

Table 5 Components of milk/dairy promoting mTORC1-driven hepatocancerogenesis

Dairy component	Mode of action
Casein proteins	Hepatic synthesis of IGF-1 stimulating AKT-mTORC1 signaling
Whey proteins	Increased synthesis of insulin stimulating AKT-mTORC1 signaling
Milk branched-chain amino acids	Activation of mTORC1
Milk glutamine	Increasing intracellular leucine uptake stimulating mTORC1
Milk fat gobule palmitate	Palmitate-mediated activation of mTORC1
Milk exosomal MFG-E8	Promotes PI3K/AKT/mTORC1 signaling
Milk exosomal miR-21	Activates IGF-1-PI3K-AKT-mTORC1 signaling via targeting IGFBP3, PTEN, PDCD4
Milk exosomal miR-155	Activates IGF-1-PI3K-AKT-mTORC1 signaling via targeting IGFBP3, PTEN and FBXW7
Milk exosomal miR-29b	Inhibits DBT of BCKDH enhancing BCAA-mediated mTORC1 activation
Exosomal TGF-β	Accelerates epithelial-mesenchymal transition
Dairy contaminated aflatoxin M1	Increases hepatocyte BCAA levels activating mTORC1
Milk androgens	Increased miR-21 expression activating mTORC1
BMMF1 and BMMF2	Increase of hepatocyte proliferation?

mTORC1, mechanistic target of rapamycin complex 1; IGF-1, insulin-like growth factor-1; MFG-E8, milk fat globule-EGF factor 8 (lactadherin); PI3K, phosphatidylinositol-3 kinase; IGFBP3, IGF binding protein 3; PTEN, phosphatase and tensin homolog; PDCD4, programmed cell death 4; FBXW7, F-box and WD40 domain protein 7; DBT, dihydrolipoamide branched-chain transacylase; BCKDH, branched-chain α-ketoacid dehydrogenase complex.

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