Publications

Clinics of Oncology

Review Article

Lipocalin 2 as A Potential Diagnostic and/or Prognostic Biomarker in Prostate, Lung and Liver Cancer

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Volume 1 Issue 3 - 2018
Received Date: 10 June 2018
Accepted Date: 05 July 2018
Published Date: 13 July 2018

2. Keywords

Tumor microenvironment; Carcinogenesis; Matrix-metalloproteinase; Hepatocellular carcinoma; Cholangiocarcinoma; Biomarker

1. Abstract

Lipocalin 2 (LCN2) is a 25 kDa secreted protein, initially purified from neutrophil granules, and mainly expressed in immune cells, hepatocytes, renal cells, prostate, cells of the respiratory tract and cardiomyocytes. LCN2 belongs to the family of lipocalins known for their ability to traffic small hydrophobic molecules such as lipids and retinoids. Due to its ability to sequester iron-containing bacterial siderophores, LCN2 plays an essential part of the innate immunity as well as the regulation of the cellular iron metabolism. Altered LCN2 expression occurs in diverse pathological conditions, including kidney disease, obesity, steatohepatitis, inflammation and malignant transformation. In recent years, LCN2 gained attention as a potential biomarker in cancer as it is protease resistant and thus easily detectable in blood, urine, tissue and other body fluids. In line, numerous peer-reviewed reports established the LCN2 overexpression in cancers of diverse histological background. Apparently, LCN2 has contradictory roles in different types of cancers. While it facilitates tumorigenesis by promoting survival, growth, invasion and metastasis, other studies report a negative correlation of LCN2 and disease outcome. Particularly, LCN2 suppression promoted cell proliferation, migration, invasion as well as the switch from epithelial to mesenchymal state. The underlying molecular mechanisms of the complex and ambiguous role of LCN2 in malignant tumor development and progression are not completely clarified yet. The following review focuses on major findings of LCN2 as a potential diagnostic and/or prognostic biomarker in prostate, lung and liver cancer, representing worldwide three of the cancers with the highest estimated incidence, mortality, and prevalence. In addition, we will highlight the progress of knowledge in understanding molecular pathways and regulation processes of LCN2 in those cancer types.

3. Abbreviations Used

2DG, 2-desoxy-D-glucose; BALF, bronchoalveolar lavage fluid; CCA, cholangiocarcinoma; CRPC, castration-resistant prostate cancer; ELISA, enzyme-linked-immunosorbent assay; EMT, epithelial-to-mesenchymal transition; ER, endoplasmic reticulum; FAK, focal adhesion kinase; GLI, glioma-associated oncogene; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; LCN2, Lipocalin 2; MMP-9, matrix-metalloprotease-9; NGAL, neutrophil-gelatinase-associated lipocalin; NGF, nerve growth factor; NSCLC, non-small cell lung cancer; PCa, prostate cancer; PSA, prostate-specific-antigen; ROS, reactive oxygen species; S2RP^{grmc1}, sigma-2-receptor/progesterone receptor membrane component 1; SCLC, small-cell lung cancer; VDR, vitamin D receptor; qRT-PCR, quantitative real time-PCR.

4. Introduction

Lipocalin 2 (LCN2) belongs to the lipocalin protein family, a large group of proteins being involved in a diversity of biological functions. The term 'lipocalin' leads back to their functions in transporting small lipophilic molecules. This is enabled through its highly conserved three dimensional fold, composed of a single-eight-stranded continuously anti-parallel β-barrel, which forms an integral hydrophobic pocket, to carry various sub-

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Citation: Schröder SK, Asimakopoulou A, Weiskirchen R.Lipocalin 2 as A Potential Diagnostic and/or Prognostic Biomarker in Prostate, Lung and Liver Cancer. Clinics of Oncology. 2018; 1(2): 1-14

strates, including retinoic acid, fatty acids, prostaglandins, and pheromones [1,2] Details of the structure and the characteristics of the members of the lipocalin family and their diverse functions were previously reviewed by our group [3].

Physiologically, LCN2 plays an important role in cellular iron metabolism. Yang and coworkers proved that LCN2 delivers iron into the cytoplasm, where it regulates iron-associated genes, thereby controlling iron-dependent cellular reactions [4]. With regard to its ability to sequester iron-binding bacterial siderophores, LCN2 prevents bacterial access to iron and therefore decreases bacterial growth. Thus, LCN2 is also known as siderocalin [5]. An *in vivo* report consistent with the activity of LCN2 as a bacteriostatic agent showed that mice lacking LCN2 exhibit greater susceptibility to bacterial infections [6]. Other findings highlight its pivotal role in bacterial immune response, due to expression in different tissues, which are prone to microbiological infections like the lung or trachea as well as gingiva [7,8]. Beside its physiological role as a nutrient, iron strongly influences the survival of cancer cells and their metastatic spread. LCN2 has been reported to be involved in the cancer-associated iron uptake, storage and regulation [9]. Most recently, tumor-associated macrophages were proposed to secrete LCN2 into the tumor microenvironment, thereby increasing the cellular iron concentration promoting metastatic spread [10].

LCN2 is often referred as neutrophil-gelatinase-associated lipocalin (NGAL), because it was first isolated from human neutrophils [11]. The 25-kDa LCN2 is a secreted protein, which forms a complex with matrix metalloprotease-9 (MMP-9) [12,13]. Yan and coworkers found that the LCN2/MMP-9 complex is involved in extracellular remodeling due to their finding that LCN2 protects MMP-9 from auto degradation and thereby preserving its activity [14]. This interaction attracts great attention in cancer research, because MMP activity is known to enhance metastatic spread [15]. Recently, a meta-analysis confirmed LCN2 in plasma and urine as a suitable biomarker for prognosis of human colorectal and breast cancer [16].

Several other studies reported that LCN2 is able to promote tumor cell proliferation and invasion [17-19]. Cancer development and progression are complex multistep processes affecting gene expression, cell morphology, and leading to malignant behavior, including self-renewal capacity, resistance to apoptosis and their potential to infiltrate the surrounding tissue and metastasize [20].

Epithelial-to-mesenchymal transition (EMT) is called the process when epithelial cells lose polarity and cell adhesion characteristics, while transforming to cells with mesenchymal traits. At the cellular and molecular level, the loss of cell adhesion during EMT is transcriptionally mediated by suppression of E-Cadherin and various transcription factors (e.g. Snail, Slug, Zeb2, and Twist). Conversely, there is an enhanced expression of mesenchymal markers, including fibronectin and vimentin, as well as MMPs [21] correlating with invasion and metastasis [22]. The molecular pathways by which LCN2 contributes to cancer are not fully understood yet, but it seems to affect the EMT process. The upregulation of LCN2 is associated with metastasis and invasion resulting in poor prognosis. This is reported for instance in gastric cancer [23], breast cancer [24], esophageal and squamous cell carcinoma [25]. Nevertheless, there are reports illustrating a negative correlation of LCN2 in cancer progression, including colon cancer [26], pancreatic cancer [27], hepatocellular carcinoma (HCC) [28], thyroid cancer [29] and ovarian cancer [30]. These contradictory studies sometimes even observed within the same type of cancer indicate that further investigations are necessary to understand the ambiguous role of LCN2 in cancer cell progression. So far it is known, that LCN2 is involved in several pathways, including PI3K/PTEN/AKT, EGFR/STAT1, MAPK, and NF-κB signaling which play critical roles in cancer development [28,31-34]. Here we will discuss the role of LCN2 in three common types of cancer (prostate, lung, liver), including clinical findings and underlying cancer-related-pathways.

5. Cancer in Numbers

According to the latest report of the International agency for Research on cancer and the World Health Organization, more than 14 million new cases were reported worldwide in 2012 [35-37]. It is estimated that these numbers will increase to 19 million cases per year by 2025. Highest cancer incidence is found in Australia/New Zealand, South America, and Europe. From all recorded cancer types, more than 1,825,000 were lung cancers with 1,600,000 deaths, 1,112,000 prostate cancers with 307,000 deaths and 782,000 liver cancers with 745,000 deaths [35,36]. In 2012 lung cancer mortality reached 19.4 % of total deaths in cancer patients, prostate 6.6 % of the total man cancer deaths, while liver cancer mortality reached 9.1% [36]

6. LCN2 and Prostate Cancer

Prostate cancer (PCa) belongs to the four most common types of cancer worldwide [37]. There are several treatment options possible when PCa is diagnosed at an early stage of disease. However, radical prostatectomy, radiation therapy and androgen-deprivation treatment, are only effective in the absence of metastases [38].

Prostate-specific-antigen (PSA), a protein produced by the prostate, is often used in PCa screening. However, the expression of PSA also increases under non-cancerous circumstances and requires biopsies, indicating the need for novel accessible diagnostic

biomarkers [39]. Moses and coworkers found that MMPs, already known predictors of cancer [40], form a complex with LCN2, that is detectable in the urine of patients with different types of cancer, including PCa [14,40]. A decade ago higher levels of the complex of LCN2 and MMP-9 had been reported in patients with prostate cancer [41]. There are limited reports about the clinicopathological correlation of LCN2 in PCa but recently LCN2 in PCa attracted more attention. Elevated levels of LCN2 protein and mRNA have been detected in tissue and serum of patients with PCa using immunohistochemistry (IHC), quantitative real time-PCR (qRT-PCR) and enzyme-linked-immunosorbent assay (ELISA) [14,41-43]. The major studies in which LCN2 expression was investigated in PCa are mentioned in **Table 1**.

Table 1: LCN2 in human Prostate Cancer

Assay/ Method	Specimen	No. cancer patients/ samples	LCN2 expression	LCN2 and clinicopathological features	Refs
WB	urine	NS	HMW urinary MMP-9/LCN2 complex (~125 kDa)	NS	14
Chromatography, zymography, MS	urine	109	Tumor-specific fingerprint based on MMPs detected /including MMP-9/LCN2 complex	NS	41
IHC	tissue	92	High levels of LCN2 in PCa tissue, barley expression in neoplastic tissue	High LCN2 associated with degree of differentiation and Gleason's grade	42
qRT-PCR	tissue	16 (N0), 20 (N1+2)	Elevated LCN2 mRNA in different stages of lymph node metastasis in PCa	LCN2 in tissue correlates with clinical advanced disease stages	43
ELISA	serum	15 (N0) 20 (N1+2)	LCN2 is highly secreted in human serum in different stages of lymph node metastasis in PCa	Secreted LCN2 positively correlates with advanced disease stages	
qRT-PCR	tissue	48 (PCa) 10 (CRPC)	mRNA in tissue is significantly upregulated in CRPC compared to PCa	NS	55
IHC	tissue		LCN2 is expressed in cytoplasm, enhanced immunoreactivity of LCN2 in CRPC	NS	
ELISA	serum		CRPC serum exhibit significant more LCN2 than PCa serum	NS	
ELISA	serum	45	Higher values of LCN2 detected in PCa compared to BPH	LCN2 is a more specific marker to distinguish PCa from BPH than PSA (sensitivity fixed to 80%)	46
Zymography, WB	Urine	2	LCN2 and LCN2/MMP-9 complex detected in urine from PCa patients	NS	49

Abbreviations used are: BPH, benign prostatic hyperplasia; CRPC, castration-resistant-prostate-cancer; ELISA, enzyme-linked-immunosorbent assay; HMW, high molecular weight; IHC, immunohistochemistry; LCN2, lipocalin 2; MS, mass spectrometry; MMP-9, matrix metalloproteinase-9; NS, not specified; PCa, prostate cancer; PSA, prostate-specific antigen; qRT-PCR, quantitative real time polymerase chain reaction; WB, Western blot.

In contrast, relatively low expression of LCN2 was found in healthy prostate epithelial cells and glandular secretion [44,45]. Recently, LCN2 was suggested as a promising diagnostic marker for PCa screening, because it is more specific than PSA levels to distinguish PCa from benign prostatic hyperplasia (BPH) [46]. Two studies showed that LCN2 levels in human tissue and serum samples could be used to predict disease progression, because expression of LCN2 was found in lymph-node metastatic stage [43], while elevated LCN2 in human prostate cell lines was associated with the degree of differentiation and Gleason's grade in PCa suggesting that LCN2 could be a valuable marker of prostate cancer progression [42]. PCa cell lines express LCN2 in cytoplasm and secrete it to the surrounding medium. Strikingly, high-grade PCa cell lines with high metastatic potential like PC3 or DU145 express significantly higher levels of LCN2 than low grade prostate cancer cell lines such as LNCaP [47].

To establish novel therapeutic approaches and understand the mechanism of action, LCN2 signaling and regulatory relations in cancer progression have been investigated. A study of Chappell and coworkers focused on LCN2 and acquired doxorubicin resistance in prostate and breast cancer cell. They found increased LCN2 expression and secretion in drug-resistant PCa cells (e.g. PC3/Dox^{R*}) [48]. Mahadevan et al. [31] observed that human as well as murine prostate cancer cell lines significantly upregulate LCN2 after physiologically and pharmacological induction of endoplasmic reticulum (ER) stress, respectively [31]. Mechanistically, LCN2 was upregulated in NF-κB dependent manner, while pro-inflammatory cytokines were enhanced as well [31]. These results are in line with a recently published study of Chappell and colleagues, which demonstrated that LCN2 expression is positively regulated by NF-κB in human PCa cell lines [49]. In addition, inhibition of wild type p53 expression leads as well to an enhanced LCN2 expression in those cells. Upregulation of

LCN2 was associated with elevated expression of EMT markers and increased motility of PCa cells in soft agar, emphasizing the important role of LCN2 in invasion and metastasis [49].

The correlation between LCN2 and ER stress through the PI3K/AKT pathway, as a result from unfolded protein response, was analyzed in breast cancer [50]. Nerve growth factor (NGF) is upregulated in the urine of PCa patients and discussed as a biomarker for PCa [51]. By using cDNA microarray analysis, Sigala et al. [52] examined the profile of NGF-regulated genes in the PCa cell line DU145. They reported several genes which are involved in invasion and metastasis, cellular metabolism as well as in proliferation and apoptosis. Interestingly, LCN2 was downregulated (5.6-fold) after NGF treatment, which may indicate an interaction of both proteins in PCa [52].

Glioma-associated oncogene (GLI) is involved in the canonical hedgehog signaling, and a target of the S6K1 kinase. GLI1 and GLI2 are highly expressed in androgen-independent, metastatic PCa cell lines (DU145 and PC3) when compared to androgen-dependent LNCaP cells. LCN2 transcription is upregulated in LNCaP-GLI1 cells similarly to DU145 and PC3, suggesting a relation between GLI1 and LCN2 [53]. However, further investigations are needed to understand the functional connection of both proteins.

Ding and colleagues reported that LCN2 induces EMT in PCa cells through the ERK/SLUG signaling axis, whereas PTEN/AKT signaling, which activates SLUG expression in PCa as well [54], was not involved [43]. Mechanistically, overexpression of LCN2 promotes metastatic potential of PCa cells as indicated by high level expression of cyclin D1, decreased p21 quantities and increased enzymatic activity of MMPs [42]. *In vivo* a knockdown of LCN2 in a xenograft mouse model resulted in reduced tumor growth [42] and decreased pulmonary metastasis [43].

In a subsequent study, Ding and coworkers reported that LCN2 not only plays a role in PCa but also in castration-resistant prostate cancer (CRPC), also known as hormone-refractory prostate cancer, which is characterized by progressively rise of PSA in serum, the development of persisting disease, and/or occurring of new metastasis [55]. Overexpression of LCN2 in human CRPC cell lines resulted in enhanced proliferation, whereas a knockdown of LCN2 acts conversely. These results were in line with xenograft models showing a prominent increase tumor growth after injection of LCN2-overexpressing CRPC cells *in vivo*.

From the clinical perspective, the detection of elevated levels of LCN2 at aggressive stages in CRPC and PCa patients opens new approaches for early diagnosis and development of novel treatment strategies. To sum up, present studies strongly indicate a positive correlation of LCN2 expression in the progression and invasion of PCa. In Figure 1 diagnostic measures for PCa, current treatment schemes and proposed roles of LCN2 in diagnosis or therapy are depicted.

7. LCN2 in Lung Cancer

Lung cancer is worldwide the most common cancer [36]. The two main types of lung cancer are small-cell lung cancer (SCLC) and the most frequent non-small-cell lung cancer (NSCLC). SCLCs are the most aggressive form of the disease which can lead earlier to metastasis than NSCLC [56]. More than 95% of SCLC patients in USA and Europe are current or ex-smokers [57] The high mortality of lung cancer is molecularly based on the resistance of the tumor cells to initiate programmed cell death, in particular in lung adenocarcinoma. Often cancer therapies fail due to the development of resistance against cancer treatment [58]. Thus, it is mandatory to understand the cellular and molecular mechanisms of acquired resistance to improve therapeutic regimens.

Healthy lung tissue was found to express moderate LCN2 levels [44,45]. Several studies in recent years investigated the role of LCN2 in lung cancer. LCN2 is suggested as a therapeutic target for patients with lung adenocarcinoma, due to its elevated expression in this disease as visualized by IHC, Western Blot, qRT-PCR and ELISA [44,59-66]. A meta-analysis of Candido and coworkers confirmed an upregulation of LCN2 in adenocarcinoma [67]. Major recent findings of LCN2 expression in human lung cancers as well as clinicopathological features are summarized in **Table 2**.

Table 2: LCN2 in Human Lung Cancer

Assay/ Method	specimen	No. cancer patients/ samples	LCN2 expression	LCN2 and clinicopathological features	Refs
IHC	tissue	NS	High levels of LCN2 found in lung AD; squamous cell and large cell carcinomas weakly express LCN2 or are negative for LCN2	NS	44
Microarray, qRT-PCR	tissue	17	LCN2 is upregulated in metastasis group of human SqCC as compared with non-metastatic group	LCN2 is associated with metastasis/cancer progression	69
IHC	tissue	23	19/23 cases of lung carcinoma were positive for LCN2; diffuse cytoplasmic distribution	NS	60
ELISA	plasma	107	Increased level of plasma LCN2 in the progressive disease group vs. partial response to erlotinib	LCN2 may be involved in therapy response to erlotinib (NSCLC)	61
ELISA	plasma	23	NSCLC patients with EGFR mutations exhibited lower LCN2 levels compared to healthy subjects	NS	
IHC	tissue	NS	LCN2 expressed in lung AD, SqCC and surrounding areas of mucinous invasive adenocarcinoma	NS	
IHC	tissue	41	39/41 samples were positive for LCN2, samples negative for MMP-9 (10/41) were also negative for LCN2	LCN2 greater equal 70 % confers worse prognosis; independent (from MMP-9) marker	62
IHC qRT-PCR	tissue	Meta-analysis	Upregulation of LCN2 mRNA and protein in lung AD	NS	67
IHC qRT-PCR	tissue	35	Upregulation of LCN2 in lung AD *vs.* control lung	NS	63
ELISA	serum	67	Serum LCN2 levels were significantly higher in NSCLC patients	Serum LCN2 levels higher in progressed TNM stages	64
IHC	tissue	12	LCN2 was highly expressed in lung AD cases (12/12)	LCN2 as a serological biomarker	65
ELISA WB	serum	20	Significant upregulation of LCN2 in lung AD		
Lable-free LC-MS/MS	BALF	26	LCN2 in BALF increased in SqCC *vs.* control	NS	68
ELISA	plasma	46	LCN2 expression was significantly higher in lung AD and SqCC *vs.* control	NS	
ELISA	plasma	40+48	LCN2 levels after neoadjuvant chemotherapy were reduced, more distinct decrease in observation group	NS	66

Abbreviations used are: AD, lung adenocarcinoma; BALF, bronchoalveolar lavage fluid; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked-immunosorbent assay; IHC, immunohistochemistry; LCN2, lipocalin 2; LC-MS/MS, liquid chromatography-mass spectrometry; NS, not specified; NSCLC, non-small cell lung cancer; SqCC, squamous cell carcinoma; qRT-PCR, quantitative real time polymerase chain reaction; WB, Western blot.

An effective method to characterize changes in the protein signature of patient is the proteomic analysis of bronchoalveolar lavage fluid (BALF) from patients with lung cancer using label-free mass spectrometry. Levels of LCN2 in BALF were enhanced in squamous cell carcinoma compared to control samples [68]. Verification analysis of plasma samples showed significantly increased LCN2 levels in squamous cell carcinoma as well as in adenocarcinoma. The authors suggested groups of different proteins as biomarkers for NSCLC, because of the wide heterogeneity in cancer. In addition, the authors found elevated levels of Cystatin C and TIMP-1 in BALF and plasma analysis. Most importantly, the protein profiles of BALF are simply transferable to blood samples, offering a non-invasive diagnosis method [68].

LCN2 has also been found to be expressed in squamous cell carcinoma and in the surrounding regions of lung cancer cells in mucinous invasive adenocarcinoma [61]. While analyzing different types of lung cancer tissue, Zhang and coworkers detected high levels of LCN2 in lung squamous carcinoma and adenocarcinoma [60]. In a genome-wide microarray analysis, a comparison of primary squamous cell carcinoma patients with or without subsequent distant metastasis LCN2 was identified as constantly upregulated in the metastatic group and was further validated by qRT-PCR as a potential biomarker for metastatic spread in human squamous cell lung carcinoma [69].

In a more recent study, LCN2 was suggested as a serological biomarker for lung adenocarcinoma by combining proteomics and bioinformatics approaches [65]. Serum samples of 67 patients with the diagnosis NSCLC were analyzed and a significantly upregulated LCN2 was detected in comparison to the healthy control group, stressing that higher stages in the TNM classification of malignant tumours notation system correlate with an increase in LCN2 expression. In the same study, abnormal mRNA expression of genes associated with proliferation, apoptosis and invasion of tumors was found in patients with surgical removed

NSCLC lesions [64].

Some of the underlying molecular pathways, explaining the role of LCN2 in lung cancer and metastatic spread have been proposed. Oxidative stress seems to be important in LCN2 signaling. It was found that depletion of LCN2 induces production of reactive oxygen species (ROS) via the NRF2/HO-1 signaling pathway, leading to initiation of apoptosis of lung adenocarcinoma cells. In line, induced apoptosis by down regulation of LCN2 could be blocked by pretreatment with N-acetyl-L-cysteine, a common ROS scavenger. In a xenograft nude mouse model, LCN2 down regulation reduced the size and the weight of the tumor significantly by inducing apoptosis [63]. The role of LCN2 in ER stress was studied in lung cancer cells. Hsin and coworkers demonstrated that LCN2 is a new target gene of GADD153 in ER stress (e.g. induced by thapsigargin) in the immortalized A549 lung adenocarcinoma cell line [70]. Silencing of GADD153, an important inducer of apoptosis, leads to downregulation of LCN2 and conversely shRNA mediated knockdown of LCN2 reduced thapsigargin-mediated cell death in A549 cells [70].

In general, it is ambiguous whether LCN2 acts pro-or anti-apoptotic and a more comprehensive discussion can be found in [71]. Tong and coworkers found that LCN2 is activated in response to apoptosis (induction via PDK1 inhibitors), where it acts as a survival factor to protect A549 cells from apoptosis [17]. Treatment with antisera against LCN2 induced apoptosis and conversely overexpression of LCN2 in A549 cells reduced the cell death by 50% [17]. Their findings are supported by their subsequent studies in mice showing that the LCN2 orthologue, known as 24p3, is as well an MK886-inducible gene playing a crucial role in MK886-induced apoptosis [72].

Shiiba et al. [73] found that LCN2 down regulation in A549 cells (and oral cancer cell lines) led to enhanced radiosensitivity [73]. Tyrosine kinase inhibitors, like erlotinib, are effective in the therapy of NSCLC, but very often patients acquire resistance during therapeutic treatment [61]. Interestingly, the suppression of LCN2 leads to an increased erlotinib sensitivity *in vitro* and *in vivo*. In NSCLC, LCN2 negatively regulates Bim protein and thus facilitates resistance against erlotinib. Mechanistically, these results indicate that LCN2 enhances ERK signaling resulting in down regulates of the Bim protein and promotion of cell survival. Lung cancer patients, who underwent erlotinib treatment, responded better to treatment when baseline plasma LCN2 levels were lower [61].

Another study supported the involvement of AKT and ERK pathways in LCN2 signaling induction. As invasion is one of the most crucial steps in the process of tumor progression, signaling pathways leading to the spreading of cancer are important targets. It is known that Sigma-2-receptor/progesterone receptor membrane component 1 ($S2R^{Pgrmc1}$) is involved in cancer progression, especially in lung cancer. It stimulates proliferation, the metastatic process *in vitro* as well as the tumor growth *in vivo* [74] and interacts with EGFR [75]. Mir and coworkers proposed a model in which $S2R^{Pgrmc1}$ regulates LCN2 expression via EGFR in a NF-κB-dependent manner [76]. Nevertheless, EGFR overexpression in $S2R^{Pgrmc1}$ knockdown cells did not entirely restore LCN2 levels. Thus, the authors deduced that LCN2 transcription is only partially affected by EGFR signaling and other pathways e.g. HDAC1 signaling might be involved. They further speculated that inhibitors of $S2R^{Pgrmc1}$ may provide targeting strategies against elevated quantities of LCN2 in tumors [76].

The mentioned study of Zhang and coworkers underlines the importance of NF-κB signaling in lung adenocarcinoma cells. Basal activity of LCN2 transcription was regulated by a segment of the LCN2 promoter at position -152 and -141 that is critically regulated by binding of C/EBPβ [60]. Other studies demonstrated that LCN2 is induced by Interleukin-1β via NF-κB and IkB in A549 cells [77].

A comprehensive study investigated a potential function of LCN2 in EMT in lung adenocarcinoma *in vitro* [78]. Additionally, they used a 2-desoxy-D-glucose (2DG)-guided *in vivo* model, representing an excellent technique to visualize tumor formation and progression in real time. In this study, a genetic approach in which LCN2 was cloned in A549-tumor initiating cancer stem-like cells was able to enhance the process of EMT. Twist and Snail2 expression as well as MMP-9 seem to be important in the NF-κB-mediated LCN2 upregulation. Interestingly, a novel NF-κB inhibitor, BRM270, was able to revere EMT transformation *in vitro* as well as *in vivo*. Molecular imaging using 2-DG guided probes detected reduced metastatic spread in the BRM270-treated LCN2-A549-tumor initiating cancer stem-like cells and significantly decreased tumor weight [78].

In summary, most of these findings highlight the pivotal role of LCN2 as a potential biomarker in the diagnosis of lung cancer. A summary illustration of possible functions of LCN2 in therapy in human lung cancer is given in Figure 2. If referring the recent literature, there is strong evidence that LCN2 involved in the process of lung tumor progression. Therefore LCN2 is getting attention as a molecular target in lung cancer treatment. Nevertheless, further studies are urgently needed to clarify the underlying molecular mechanisms and the inconsistent findings concerning its role in survival and apoptosis.

8. LCN2 in Primary Liver Cancer

8.1. LCN2 in hepatocellular carcinoma

HCC is the most common type of primary liver cancer and one of the major causes of cancer-related death around the world

[36,79]. There are a wide variety of risk factors with a strong association to the development of HCC. The most common factors, further discussed by Ghouri and colleagues [79], are viral infections (hepatitis B and hepatitis C), alcoholic as well as non-alcoholic fatty liver diseases, and mycotoxins such as aflatoxin. Chronic infections, metabolic stress or toxic damage can lead to a persistent hepatic inflammation. In around 90% of the cases, HCC occurs due to underlying cirrhosis [80].

Our laboratory investigated the role of LCN2 in inflammatory hepatic injury. We have shown that injured hepatocytes are the major source of LCN2 in diseased liver [81-83]. LCN2 is a promising biomarker for detection of early stages of hepatic damage in injured liver and seems to protect the liver [81-83]. Currently, α-fetoprotein is used for diagnosis of HCC associated with inflammation, however, it fails to detect early stages of cancer [84]. To improve cancer therapy one of the most important steps is to reveal the underlying molecular pathways and to detect novel biomarker indicating the disease at an early stage. The role of LCN2 in the initiation and progression of HCC seems to be complex and still not fully understood. Altered LCN2 expression in the pathogenesis of HCC was found in several studies *in vivo* and *in vitro* studies. Some of the major studies in which LCN2 was investigated in human HCC are summarized in **Table 3.**

Table 3: LCN2 Expression in Human HCC

Assay/ Method	specimen	No. cancer Patients/ samples	LCN2 expression	LCN2 and clinicopathological features	Refs
Oligo-nucleotide array	tissue	7	LCN2 upregulation in HCC	LCN2 upregulation (2.8-fold) in progressed 'nodule-in nodule' HCC *vs*. early stage	86
DNA microarray	tissue	82	LCN2 upregulation (8.07-fold) in HCC	NS	87
RT-PCR	tissue	8	LCN2 highly expressed in 5/7 tumor samples	NS	
ISH	tissue	NS	LCN2 highly expressed in malignant hepatocytes	NS	
NB	tissue	15	LCN2 mRNA higher in 11/15 HCC tissues	NS	28
Microarray, IHC	tissue	NS	LCN2 positive in infiltrated cells and liver tumor cells	NS	
WB	tissue	16	LCN2 upregulated only (> 1.5-fold) in 2/16 HCC; LCN2R upregulated only (> 1.2-fold) in 4/16 HCC	NS	88
IHC	tissue	138	LCN2 was highly expressed in 102/138 HCC, LCN2R was highly expressed in 95/138 HCC, combined expression in 92/138 HCC	LCN2 and LCN2R are higher expressed in higher TNM stages, correlation with vascular invasion and tumor recurrence, high LCN2R expression correlates with poor prognosis	89
ELISA	serum	25	LCN2 levels were higher in HCC patients *vs*. healthy control	Higher LCN2 expression in patients with HCC on top of HCV *vs*. HCV patients, LCN2 is indicative for disease progression	91
DNA microarray	tissue	42	LCN2 were higher (2.05-fold) expressed in HCC tissue	LCN2 upregulated in well-differentiated HCC	33
RT-PCR	tissue	40	25/40 HCC expressed higher LCN2	LCN2 correlate with worse differentiation grade and poor prognosis, LCN2 negatively correlates with EMT	
WB, IHC	tissue	12	High LCN2 expression in 58% of HCC tissue	Increased LCN2 expression in various tumor stages	
qRT-PCR	tissue	80	LCN2 overexpressed in 57/80 HCC, TRα higher in 61/80 cases	Correlation between LCN2 and TRα and association with poor patient survival	90
Aptamer-based sandwich assay	serum	4	Ability to detect LCN2 in clinical samples was comparable to ELISA, concentration range of 2.5-500 ng/ml for LCN2 detection	Suitable methods to detect LCN2 in clinical relevant serum ranges	94
ELISA	blood	54	LCN2 was significantly higher in HCC patients	Correlation of LCN2 and various clinical parameters, decreased survival rate in patients with LCN2 levels above 119 ng/ml	92
qRT-PCR	tissue	40	non-responding (sorafenib) liver cancer patients had higher LCN2 expression	Better therapy response to sorafenib with decreased LCN2 level	34

Abbreviations used are: ELISA, enzyme-linked-immunosorbent assay; EMT, epithelial-to-mesenchymal transition; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; ISH, in situ hybridization; LCN2, lipocalin 2; LCN2R, lipocalin 2 receptor; NB, Northern blot; NS, not specified; TRα, thyroid-hormone receptor α; qRT-PCR, quantitative real time polymerase chain reaction; WB, Western blot.

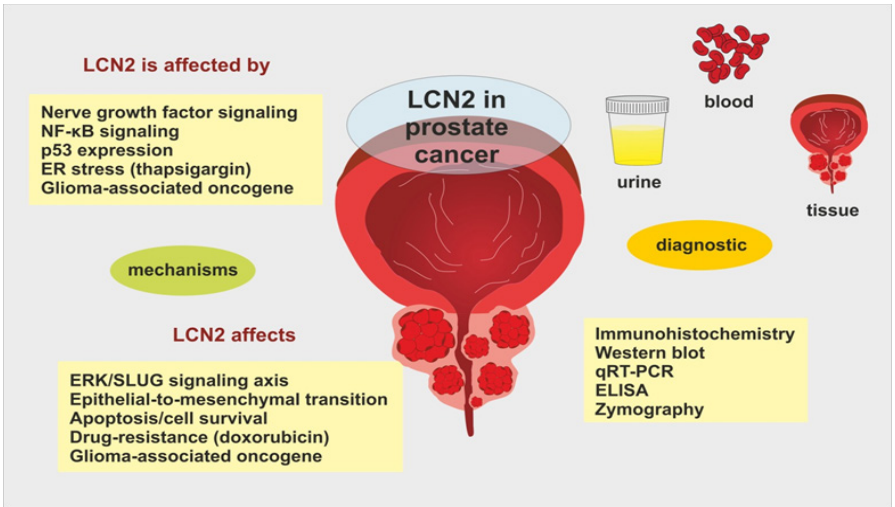

Figure 1: Diagnostic measures, modulator factors, and proposed functions of Lipocalin 2 in pathogenesis of prostate cancer. In the pathogenesis of prostata cancer, LCN2 expression is modulated by different pathways and factors (Nerve growth factor, NF-κB, p53 oncogene, endoplasmic reticulum stress, glioma-associated oncogene). Altered expression of LCN2 impacts ERK/SLUG signaling, epithelial-to-mesenchymal transition, apotosis/cell survival, drug resistance, expression of glioma-associated oncogene. In diagnosis of prostata cancer altered quantities or biological activity of LCN2 in urine, blood or tissue specimen can be measured by different methods including immunohistochemistry, Western blot, qRT-PCR, ELISA or zymography.

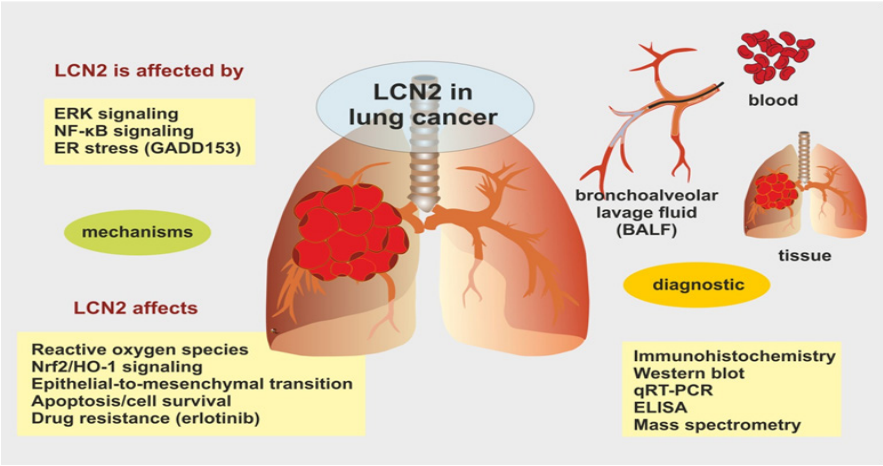

Figure 2: Diagnostic measures, modulator factors, and proposed biological functions of Lipocalin 2 in lung cancer. In lung cancer LCN2 expression was found to be modulated by ERK and NF-κB signaling as well as endoplasmic reticulum (ER) stress. Altered expression of LCN2 affects reactive oxygen species formation, Nrf2/HO-1 signaling, epithelial-to-mesenchymal transition, apoptosis/cell survival, and drug resistance. Different studies used immunohistochemistry, Western blot, qRT-PCR, ELISA, or mass spectrometry to identify altered expression of LCN2 in bronchoalveolar lavage fluid (BALF), blood, or lung tissue.

The healthy liver seems to express low quantities of LCN2 which can be further upregulated by inflammation, bacterial infections or irradiation [44,85]. However, no LCN2 levels were detected in normal hepatocytes, but found in smaller intrahepatic bile ducts [44]. High expression of LCN2 was detected by microarray analysis during expression profiling in multistage hepatocarcinogenesis in human tissue, proposing its important role in the development of HCC [33,86,87]. Besides its expression in human tissue [28,33,86-90] LCN2 was significantly upregulated in the blood of HCC patients [91-93]. There are also initial innovative concepts for detecting LCN2 in HCC through an aptamer-based sandwich assay [94].

In vivo experiments from a woodchuck animal model examining *Woodchuck* hepatitis virus (WHV)-induced HCC, demonstrated higher expression of LCN2 mRNA in HCC compared to non-tumorous tissue, but no change in protein level [95]. Besides LCN2, both MMP-9 expression and enzymatic activity were enhanced in animals with HCC, suggested being as well an important marker in HCC progression. In an N-nitrosodiethylamine-induced animal model, rats showed increased serum levels of LCN2 as other potential biomarkers of HCC [93].

One treatment opportunity of liver cancer is the use of the kinase inhibitor sorafenib. LCN2 upregulation was found in a cohort of 40 patients, who did not respond to sorafenib treatment [34]. Most likely, signaling pathways (e. g. EGFR/STAT1, MAPK, and NF-κB) activating SULF2-mediated LCN2 expression and altering sorafenib sensitivity are altered in these patients [34].

Chien and coworkers found down regulation of LCN2 and its receptor LCN2R in most of the analyzed human HCC tissue samples and only weak expression in HCC cell lines [88]. However, Zhang et al. [80] correlated the expression of LCN2 and LCN2R with unfavorable clinicopathological features [89]. This is sustained by the finding that LCN2 is upregulated (2.8-fold) in progressed components of nodule-in-nodule-type HCC but not in earlier stages [86]. The clinical significance of LCN2 was assessed in 80 consecutive HCC patients through qRT-PCR where 71.3% showed increased expression levels of LCN2, which correlated with poor survival [90]. The most recent study analyzed blood LCN2 levels in human chronic liver disease patients and found a significant upregulation of LCN2 [92]. In summary, the authors of that study disclosed that LCN2 levels above 119 ng/ml are a negative prognostic value for patient survival in chronic liver disease with HCC [92].

It's still ambiguous whether LCN2 promotes or counteracts progression and metastatic spread of cancer. In various types of cancer LCN2 is associated with the EMT process. On the one hand findings indicate that overexpression of LCN2 in HCC leads *in vitro* to a reduction of invasive capacity, migration, proliferation, and an inhibition of EMT through reduced MMP-2 expression [28]. Xenograft models confirmed the reduced proliferation activity and revealed reduced tumor growth *in vivo*. Mechanistically, LCN2 partially acts by inhibiting the PI3K/AKT and JNK signaling pathways. Besides, there is strong evidence that LCN2 negatively exerts its function via the TGF-β1/Twist pathway [33]. Knockdown of LCN2 strongly induces Twist1 expression as a marker for EMT, whereas LCN2 overexpression reduced proliferation and invasion *in vitro* as well as tumor growth and metastasis *in vivo*. Therefore, LCN2 was suggested as an early stage biomarker to detect HCC before metastatic spread [33]. Chien and coworkers reported that LCN2 can elicit apoptosis of HCC cells, marked by inhibition of cell proliferation in LCN2 over expressing cells, DNA fragmentation and cell-cycle arrest at sub-G_1-phase [88]. In the mentioned study LCN2-induced apoptosis was proposed to partially act through the mitochondria pathway, via increased Bax/Bcl-2 ratio in Huh7 and SK-Hep1 cells and depolarization of their mitochondrial membrane [88].

Oxidative Stress, especially ROS, in the tumor microenvironment influences cancer development and affects its progression. Irradiation of mice with γ-rays strongly upregulated LCN2, particularly in the liver [96]. In addition, LCN2 expression was increased in the HCC cell line HepG2 after γ-rays and H_2O_2, a common inducer of oxidative stress [85]. The same group found that LCN2 is able to protect A549 cells with ectopic expression of LCN2 against oxidative stress induced by H_2O_2 [97]. LCN2 has been found to be upregulated in different HCC-inducible mouse models, including acyl-CoA oxidase (AOX)-deficient mice. AOX is the rate limiting enzyme in peroxisomal β-oxidation, whose absence leads to increased hydrogen peroxide [98]. These findings indicate a protective role of LCN2 in HCC in suppressing the metastatic spread, which has been found as well in other types of cancer, including Ras-transformed mouse mammary *cells in vitro* [99] and ovarian cancer [30].

One key regulator in cancer is c-met directly interacting with focal adhesion kinase (FAK) through phosphorylation to promote cell migration in lung cancer cells [100]. FAK and c-met are also important proteins critically involved in HCC signaling [101]. Chung and coworkers suggested LCN2 to promote the progression of HCC spread and cancer progression via regulation of LCN2 through the Met/FAK axis [90]. In the same study, the thyroid hormone T_3 was unraveled as a critical factor playing a role in the thyroid-hormone receptor-α induction through the Met/FAK axis and upregulation of LCN2 via a thyroid hormone response element located at position -1444/-1427 of the human LCN2 promoter encompassing the sequence 5'-GGATACTTTT-TAAGGTCA-3'. The authors confirmed their findings *in vitro*, *in vivo* and furthermore in a cohort of 80 HCC patients [90].

The mentioned studies indicate that the role of LCN2 in cancer progression is rather complex. There is limited knowledge of underlying molecular pathways and regulatory networks of LCN2 in HCC. New studies targeted on the direct relationship of endogenous and secreted LCN2 in HCC are needed to clarify the possible function of LCN2 for establishment of novel therapies. According to the latest findings an illustration in **Figure 3** (upper part) aims to clarify how LCN2 participates in HCC.

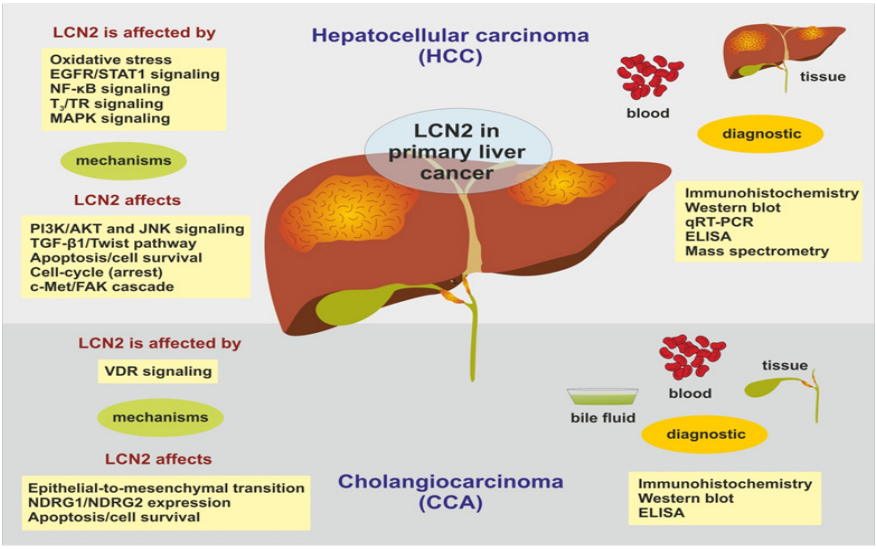

Figure 3: Lipocalin 2 in the pathogenesis and diagnostic of hepatocellular carcinoma and cholangiocarcinoma. It is proposed that LCN2 is a critical factor in the pathogenesis of both hepatocellular carcinoma and cholangiocarcinoma. It is reported that altered LCN2 expression during initiation or progression of primary liver cancers is modulated by a large numbers of pathways. Vice versa, altered LCN2 quantities impacts several pathways and genes critically involved in the pathogenesis of respective diseases. Altered LCN2 expression is detectable in blood, tissue (liver and bile duct specimens), blood, or bile fluid. A large set of methods (e.g. immunohistochemistry, Western blot, qRT-PCR, ELISA, and mass spectrometry) were applied to detect altered LCN2 mRNA or protein expression.

8.2. LCN2 in cholangiocarcinoma

Cholangiocarcinoma (CCA), or bile duct cancer, is the second common type of primary liver cancer next to HCC. Whereas HCCs evolve by malignant conversion of hepatocytes, intrahepatic CCA develops from the small intrahepatic bile duct epithelium. The development of CCA is not fully understood yet, but mostly arises *de novo* and has different risk factors [102] including the liver fluke (*Opisthorchis viverrini*) and nitrosamine intake [103]. CCA has one of the lowest survival times (~ 5 years) due to late diagnosis and resistance to general cancer treatment strategies [104].

While there are conflicting findings concerning LCN2 in HCC, there are consistent reports, indicating that LCN2 provide a promising diagnostic marker and as well a target structure for CCA therapy. Studies on LCN2 expression in CCA are mentioned on **Table 4.** High LCN2 expression was detected in tissue of CCA patients by the use of IHC staining [105-107] and immunoblotting [108]. In all studies no LCN2 expression was found in corresponding non-tumorous tissue. Chiang and coworkers reported strong expression of LCN2 in human CCA samples and a negative correlation with overall patient's survival [107]. Additionally, this study suggested LCN2 as a potential marker in diagnosis of CCA and in distinction to patients suffering from gallstone, due to increased values of LCN2 in bile in CCA patients (cut-off: 20.08 ng/ml).

Leelawat and coworkers found significantly elevated serum LCN2 levels in CCA patients compared to patients with benign biliary tract disease and higher levels of LCN2 in advanced CCA than in early CCA [109]. In contrast, another study demonstrated no differences in serum between malignant and benign biliary diseases, while LCN2 levels were enhanced in bile from intrahepatic CCA patients [110].

Wang et al. demonstrated strong LCN2 expression in CCA cell lines (Choi-CK, Cho-CK, JCK) besides LCN2 expression in HCC cell lines with epithelial phenotype [33]. Srisomsap and coworkers performed for the first time a proteomic analysis of the secretome of different HCC and CCA cell lines via LC-MS/MS analysis [108]. However, they only found LCN2 in the secretome of Hu-CCA1 cells. Seven years later, the same laboratory published further investigations on secretome analysis [111], where they used their established 3D culture system using hollow fiber bioreactor (HFB) [112]. In comparison to conditioned medium from monolayer cultures, HFB are characterized by enhanced quality and quantity [113]. In their study, LCN2 was the most highly expressed protein in MS/MS analysis in the secretome of CCA cell lines that was also validated by Western Blot analysis. These results correspond to a recent report proposing LCN2 as a CCA diagnostic marker [107].

Table 4: LCN2 Expression in Human Cholangiocarcinoma

Assay/ Method	specimen	No. cancer patients/ samples	LCN2 expression	LCN2 and clinicopathological features	Refs
IHC	tissue	24	LCN2 expression in cytoplasm of all CCA patients (24/24), several CCA showed strong expression (18/24)	No correlation with tumor differentiation, lymph-node, metastatic status	105
ELISA	serum	50	significant higher LCN2 in CCA in comparison to control	LCN2 levels are significant different between early (TNM I + II) and advanced (TMN III + IV) tumors, LCN2 increase in progressed CCA, CA19-9 and LCN2 together are promising in diagnosis showing high sensitivity	109
ELISA	serum	16	LCN2 positive in benign/malignant disease	Serum LCN2 has no significant value for discriminating between malignant and benign biliary strictures	110
	bile fluid	16	LCN2 positive in benign/malignant disease	Bile LCN2 levels are suitable to differentiate between pancreatobilary cancer and benign biliary disease, together with CA19-9 increased sensitivity	
IHC	tissue	80	53/80 positive, LCN2 localized in the cytoplasm of CCA cells	NS	106
ELISA	bile fluid	30	Significantly higher LCN2 level in CCA than in gallstone patients	NS	107
IHC	tissue	78	36/78 cases low LCN2 expression, 42/78 cases showed high LCN2 expression	LCN2 is inversely correlated with survival of CCA patients	
WB	tissue	12	12/12 cases positive for LCN2, 9/12 showed high expression	NS	108

Abbreviations used are: CA-19-9, cancer antigen 19-9; CCA, cholangiocarcinoma; ELISA, enzyme-linked-immunosorbent assay; IHC, immunohistochemistry; LCN2, lipocalin 2; NS, not specified; WB, Western blot.

Chiang et al. demonstrated that LCN2 knockdown in CCA cell lines resulted in increased cellular doubling times and reduced migration ability, underpinning the role of LCN2 role as a potential oncogene in human CCA [106]. These findings correspond to another study by Nuntagowat et al. [106] in which a knockdown of LCN2 (via siRNA technology) facilitated the invasion and migration rate of CCA cells, partly, by stabilizing MMP-9 in the complex with LCN2, preventing its degradation [105]. In addition, it was shown that the intra- and extracellular MMP-2 as well as MMP-9 expression levels were decreased in LCN2 knockdown cells in CCA, which was consistent with reduced invasion [107]. LCN2 seems to enhance EMT in CCA, due to decreased levels of transcription factors (e.g. SNAIL, TWIST), increased E-cadherin level, reduced metastatic potential in SNU308-LCN2 silenced CCA cells and inhibition of CCA tumor formation *in vivo* xenograft model. Chiang et al. found for the first time that LCN2 acts upstream of NDRG1 and NDRG2 in CCA cells, representing important tumor suppressors in the cell [107]. Moreover, the same group suggested vitamin D receptor (VDR) and LCN2 to affect each other in human CCA cell lines, because $1\alpha,25(OH)_2D_3$ treatment (an active form of vitamin D, ligand of VDR) resulted in a significant down regulation of LCN2 accompanied with dose-dependent anti-proliferation of the cells [106]. The shRNA-mediated VDR knockdown in SNU308 cells led to increased LCN2 expression. In a thioacetamide-induced CCA rat model: LCN2 mRNA as well as protein levels were suppressed when animals were supplemented with vitamin D_3. Moreover, a lower tumor incidence and progression were found [106]. In line, MART-10, an analogue form of $1\alpha,25(OH)_2D_3$, was able to reduce proliferation of CCA cell lines *in vitro* more strongly and could repress tumor growth an *in vivo* xenograft model. It was demonstrated that MART-mediated growth inhibition is partially mediated by down regulation of LCN2. This mechanism of action is partially mediated by the VDR, whose expression in CCA patients correlated positively with overall survival [114].

In summary, all studies reported showed enhanced expression of LCN2 in CCA, highlighting its potential as a promising biomarker in CCA to enable early diagnosis and initiated treatment options with vitamin D_3 analogs. It would be enlightening, if there is indeed a correlation between LCN2 expression and the advanced stage of disease. Further studies may focus on other important pathways in cancer, leading to progression of the tumor. Some of the suggested functions of LCN2 in pathogenesis of CCA are depicted in Figure 3 (lower part).

9. Conclusion

Although controversial discussed, the role of LCN2 in the progression of cancer seems to be of key functionality. It seems that LCN2 has tumor type-dependent roles in the pathogenesis of prostate, lung and liver tumorigenesis. Moreover, secreted and endogenous LCN2 levels can give information on the different stages of cancer development in each tissue. All of the discussed findings suggest that the biological functions of LCN2 are strongly influenced by the tumor microenvironment (e.g. oxidative stress or cytokine release). Most of the recent studies proposed LCN2 as a non-invasive biomarker in blood, tissue or other body fluids of cancer patients. However, to clarify ex-

pression of LCN2 in progressive disease stage, greater cohorts of human patients should be analyzed. Nowadays, several different pathways are suggested to regulate LCN2 expression in different types of cancer. Future studies are now mandatory to unravel the underlying cellular and molecular pathways of LCN2 regulation and activities in cancer development. However, it became clear during the last decade that LCN2 is a major pleiotropic protein relevant in cancer development and progression.

10. Acknowledgements

RW is supported by grants from the German Research Foundation (DFG, SFB/TRR 57, projects P13 and Q3) and from the Interdisciplinary Centre for Clinical Research within the Faculty of Medicine at the RWTH Aachen University (IZKF Aachen, Project O3-1). The authors thank Sabine Weiskirchen for preparing images for this review.

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