Increased expression of lipocalin-2 in liver tissues in chronic viral hepatitis and neoplasia

Kronik viral hepatitler ve neoplazilerde karaciğer dokusunda artmış lipocalin-2 ekspresyonu

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Background and Aims: *Lipocalin-2 is overexpressed in various inflammatory and neoplastic tissues. However, the relationship between lipocalin-2 and liver diseases has not been documented well. This study investigated the expression of lipocalin-2 in the liver of chronic viral hepatitis and neoplastic tissues.* ***Material and Methods:*** *A total of 47 (2 normal, 20 benign, and 25 malignant) liver tissues were used. Specimens from patients with chronic viral hepatitis B (n = 10) and chronic viral hepatitis C (n = 10), hepatocellular cancer (n = 15), liver metastasis of adenocarcinoma (n = 8), and neuroendocrine tumors (n = 2) were stained for lipocalin-2, and the staining intensity was graded as follows: 0, no staining; 1+, weak; 2+, moderate; and 3+, strong immunoreactivity.* ***Results:*** *Lipocalin-2 expression in the liver was increased in viral infections and was significantly augmented with the presence of fatty infiltration in both chronic viral hepatitis B and chronic viral hepatitis C. Liver metastasis of neuroendocrine tumors showed very weak staining similar to that in the normal liver. However, lipocalin-2 expression was higher in both hepatocellular cancer and liver metastasis of adenocarcinoma than in both chronic viral hepatitis B and chronic viral hepatitis C ($p < 0.001$ compared to hepatocellular cancer, $p > 0.05$ compared to liver metastasis of adenocarcinoma).* ***Conclusion:*** *Lipocalin-2 expression in the liver increases with chronic viral hepatitis, and fatty infiltration significantly augments the expression intensity. However, lipocalin-2 expression is highest in both hepatocellular cancer and liver metastasis of adenocarcinoma similarly, suggesting that lipocalin-2 increases during neoplasia independent of the type of the cell.*

Key words: *Lipocalin-2, viral hepatitis, hepatocellular cancer, metastatic liver tumors*

Giriş ve Amaç: *Lipocalin-2 çeşitli inflamatuvar ve neoplastik dokularda artmış olarak bulunmuştur. Ancak karaciğer hastalıkları ile lipocalin-2'nin ilişkisi net olarak ortaya konmamıştır. Bu çalışmada amaç kronik viral hepatitlerde ve neoplastik karaciğer dokularında karaciğer lipocalin-2 ekspresyonunu incelemektir.* ***Gereç ve Yöntem:*** *Toplam 47 (2 normal, 20 benign ve 25 malign) karaciğer dokusu incelendi. Kronik viral hepatit B (n=10) ve kronik viral hepatit C (n=10), hepatosellüler kanser (n=15), adenokarsinoma karaciğer metastazı (n=8) ve nöroendokrin tümörlerden (n=2) elde edilen karaciğer dokuları lipocalin-2 ile immünohistokimyasal olarak boyandı ve boyanma yoğunluğu; 0: boyanma yok, 1+ zayıf, 2+ orta ve 3+ güçlü immünoreaktivite şeklinde derecelendirildi.* ***Bulgular:*** *Karaciğer lipocalin-2 ekpresyonu viral enfeksiyonlarda artmış olarak saptandı ve bu artış hem kronik viral hepatit B hem de C hastalarında yağlı infiltrasyon varlığında daha belirgindi. Nöroendokrin tümörlerin karaciğer metastazında ise lipocalin-2 ekspresyonu oldukça zayıf olup normal karaciğer ile benzerdi. Öte yandan hem hepatosellüler kanser hem de adenokarsinoma karaciğer metastazında ise lipocalin-2 ekspresyonu kronik viral hepatit B ve C'ye kıyasla artmış olarak bulundu ($p < 0.001$ hepatosellüler kanser vs kronik viral hepatitler, $p>0.05$ adenokarsinoma karaciğer metasazı vs kronik viral hepatitler).* ***Sonuç:*** *Lipocalin-2'nin karaciğerde ekspresyonu kronik viral hepatitlerde artmış olup yağlı infiltrasyon varlığı artışı daha belirgin hale getirmektedir. Ancak karaciğerde lipocalin-2 ekspresyonunun en belirgin olarak hem hepatosellüler kanser hem de adenokarsinomların karaciğer metastazında olması hücre tipinden bağımsız olarak lipocalin-2'nin neoplazide anlamlı olarak arttığını göstermektedir.*

Anahtar kelimeler: *Lipocalin-2, viral hepatit, hepatosellüler kanser, metastatik karaciğer tümörü*

INTRODUCTION

Lipocalins primarily classified as transport proteins have been extensively used as biochemical markers of diseases (1, 2). Lipocalin-2 [LCN2; also known as siderocalin, NGAL (neutrophil gelatinase-associated lipocalin), uterocalin, or neu-related lipocalin], a member of the lipocalin protein family, is particularly found in specific granules of human neutrophils (2) and executes a wide variety of functions, including transport of fatty acid or iron (3) and induction of apoptosis (4). It has also been suggested to serve as a modulator of the inflammatory response based on findings such as the marked expression of LCN2 in tissues exposed to microorganisms (5, 6), its induction in murine macrophages by bacterial lipopolysaccharide (7), and the ability of LCN2 to bind lipophilic inflammatory mediators such as platelet activating factor and leukotriene B4 (8).

Expression of LCN2 has been reported primarily during bacterial infections (5-7). Nevertheless, it has also been found to be significantly upregulated in infections with

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Manuscript: 04.08.2016 • **Accepted:** 05.09.2016

viruses (9). Chronic liver diseases caused due to viral hepatitis are among the common liver diseases that can lead to serious complications. Only few studies have analyzed the possible relationship between chronic viral hepatitis and LCN2. In a recent study carried out in patients with chronic hepatitis C (CHC), urinary LCN2 levels were found to be increased (10), and it has been suggested as a marker of fibrosis. However, urinary LCN2 is not a direct reflection of the pathology in the liver and can be affected by several conditions unrelated to CHC. Moreover, there is no study performed on patients with chronic viral hepatitis B (CHB) analyzing the expression profile of LCN2 in the liver.

Chronic liver inflammation and hepatic regeneration induced in part by infection with hepatitis B or hepatitis C virus, and the consequent cellular immune responses, may increase the risk of developing hepatocellular carcinoma (HCC) by favoring the accumulation of genetic alterations in hepatocytes that might trigger specific oncogenic pathways (11). LCN2 is found to be expressed in cirrhotic human liver tissues as well as in HCC (12-14). However, it has also been reported to be overexpressed in several human cancers (1,15,16). The liver is the organ that frequently harbors the metastasis of several cancers. The pattern of LCN2 expression in the liver in different malignancies, e.g., HCC and metastatic liver cancers, has not been analyzed previously, and it is also not known whether the expression of LCN2 in the liver in malignancies is cell type specific.

The aim of the present study was to examine the expression of LCN2 in chronic viral hepatitis B and C and in different neoplastic liver tissues by immunohistochemistry.

MATERIALS and METHODS

Specimens collected from 47 patients containing malignant, viral hepatitis, and normal liver were used for immunohistochemistry. The etiology of HCC (n = 15) showed that 10 patients had CHB, 1 had CHC, and the remaining patients had an unknown etiology. All slides were reviewed by two pathologists blinded to the diagnosis.

Immunohistochemistry

Sections measuring 4 µm thicknesses were cut from paraffin blocks. The slides were dried in an oven (at 55°C-60°C) for 20 min and then loaded onto a Bench-

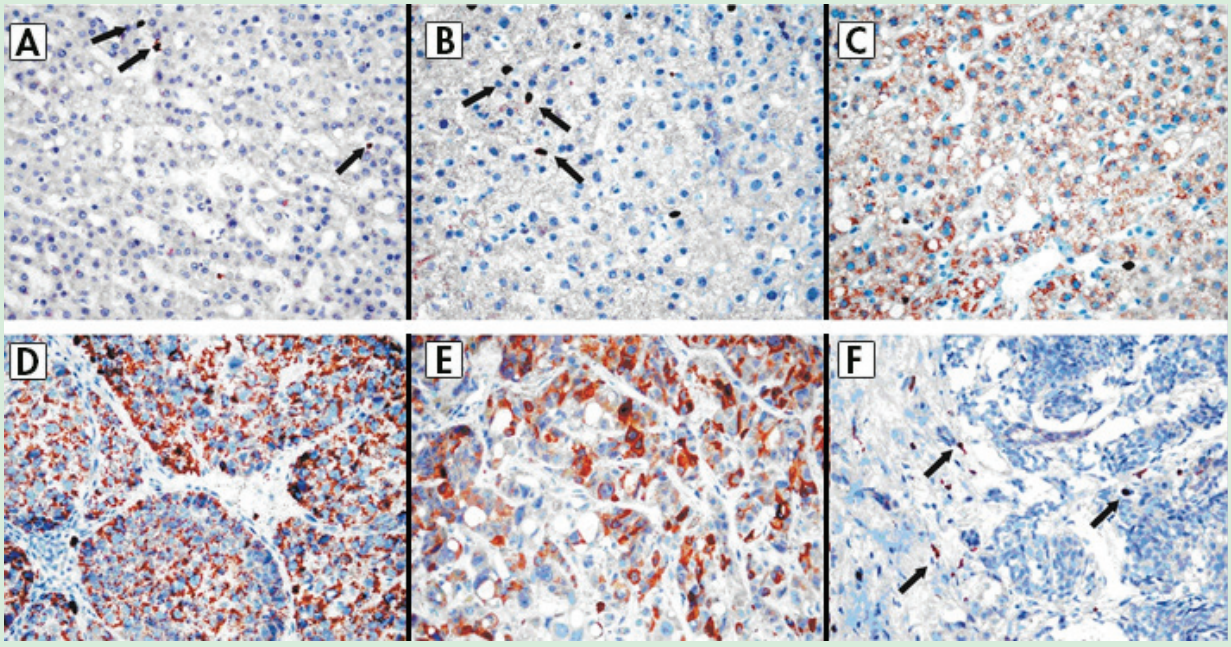

Figure 1. Immunohistochemical staining of NGAL in liver tissues. Representative fields were photographed at 400× magnification. **(A)** Normal liver tissues and neutrophils present in the stroma (arrows); **(B)** Chronic hepatitis B infection and neutrophils present in the stroma (arrows); **(C)** Chronic hepatitis C infection; **(D)** Hepatocellular carcinoma; **(E)** Metastatic liver cancer originating from pancreatic ductal adenocarcinoma; **(F)** Metastasis of neuroendocrine carcinoma of the lung into the liver tissue and neutrophils present in the stroma (arrows). Weak NGAL expression was detected in the normal liver tissue and in chronic hepatitis B infection. Moderate expression of NGAL was found in chronic hepatitis C and strong expression was detected in cancer cells and also in neutrophils (arrows). No NGAL expression was found in metastatic neuroendocrine carcinoma. NGAL: Neutrophil gelatinase-associated lipocalin.

mark instrument (Ventana Medical Systems, Tucson, AZ). Briefly, after deparaffinization and cell conditioning processes, slides were incubated with rabbit anti-lipocalin-2 polyclonal antibody (1:100 dilutions) for 36 min. The Ventana DAB basic detection system was used to visualize antibody labeling. Slides were counterstained on the instrument with hematoxylin and post-counterstained with a blue reagent. All slides were removed from the stainer and rinsed in detergent water to remove the coverslip oil. They were then mounted on a coverslip with a synthetic mounting medium after dehydration and cleared with several changes of xylene.

All slides were observed under a Nikon light microscope, and the staining intensity of immunoreactivity of LCN2 was scored and graded on a scale of 0-3+ (0 for no staining, 1+ for weak immunoreactivity, 2+ for moderate immunoreactivity, and 3+ for strong immunoreactivity). Neutrophils served as the internal control. The presence of >5% steatosis was regarded as fatty infiltration.

Statistical analysis

The Mann-Whitney U-test was used to compare the composite scores among the groups (normal liver/hepatitis/neoplastic tissues). Differences between the groups were considered statistically significant when the p value was <0.05.

RESULTS

Using the staining pattern of the normal liver tissue as a reference, we observed that the expression of LCN2 increased with viral infections, and the highest staining intensity was found in neoplastic liver tissues independent of the origin, whether primary or metastatic. Only metastasis of neuroendocrine tumors to the liver did show weak staining comparable with that in the control liver tissue.

Immunochemical staining (Figure 1)

The staining pattern of LCN2 in the liver was cytoplasmic-granular.

Normal liver (n=2): Staining of hepatocytes for LCN2 expression showed weak intensity around the central and periportal zone predominantly. In addition, neutrophils nearby the periportal zone were stained strongly as was expected.

Viral hepatitis

Chronic viral hepatitis B (n=10): The staining pattern of LCN2 in the specimens of patients with chronic viral hepatitis B was generally of weak to moderate intensity resembling that in the normal liver tissue.

Chronic viral hepatitis C (n=10): LCN2 expression was similar but slightly stronger than that in chronic viral hepatitis B, and the intensity of staining was moderate.

In both chronic viral hepatitis groups, staining of LCN2 was negative in the fibrosis areas. The staining intensity, however, tended to increase markedly with the presence of fatty infiltration ($p<0.05$).

Neoplastic liver tissues

Metastatic liver (liver metastasis of adenocarcinoma (LMA): n=8, neuorendocrine metastasis: n=2): Tumoral cells from the adenocarcinoma metastasis to the liver were generally stained strongly with LCN2. However, metastasis of the neuroendocrine tumors was stained very weakly similar to that in the normal liver tissue.

Hepatocellular cancer (n=15): All except one specimen were stained very strongly with LCN2. The staining was present primarily in the HCC cells.

Table 1. Immunohistochemical assessment of LCN2 expression in the normal, chronic viral hepatitis B and C, and neoplastic liver tissues

Tissue Specimen	N	Average Composite Number
Normal liver	2	1
Chronic viral hepatitis B	10	1.4±0.55[1,2]
Chronic viral hepatitis C	10	1.78±0.44[1,2]
Hepatocellular carcinoma	15	2.93±0.27[1,2,3]
Adenocarcinoma metastasis to liver	8	2.33±0.87[3]
Neuroendocrine tumor metastasis to liver	2	1

N: specimen number; LCN2: lipocalin-2
The staining intensity was graded on a scale of 0-3 (0=no staining, 1=weak, 2=moderate, and 3=strong staining).
[1]: p>0.05 between chronic viral hepatitis B and C.
[2]: p<0.001 between hepatocellular carcinoma and chronic viral hepatitis B or C.
[3]: p > 0.05 between hepatocellular carcinoma and adenocarcinoma metastasis to the liver.

Table 2. Immunohistochemical assessment of LCN2 expression in chronic viral hepatitis* with or without fatty infiltration

Tissue Specimen	N	Average Composite Number
Chronic viral hepatitis with fatty infiltration	13	1.77±0.44[1]
Chronic viral hepatitis without fatty infiltration	7	1.17±0.41[1]

*: either chronic viral hepatitis B (n=4.40% of CHB cases) or C (n=9.90% of CHC cases).
N: specimen number; LCN2: lipocalin-2;
The staining intensity was graded on a scale of 0-3 (0=no staining, 1=weak, 2=moderate, and 3=strong staining).
[1]: $p < 0.05$.

When the neoplastic liver specimens were compared, no significant difference was observed among the staining features of both primary and metastatic liver tumors. Liver tissue close to the tumoral tissue showed a similar staining pattern but slightly stronger than that in the normal liver tissue.

Dysplasia: There was no absolute displastic case. Nevertheless, some displastic changes were observed in chronic viral hepatitis specimens and in areas close to the tumoral tissues, and the intensity of staining was high in these areas.

LCN2 staining score of the specimens (Tables 1 and 2)

The mean score of staining intensity of LCN2 was lowest (score=1) in the normal liver. LCN2 expression in the liver tissues from patients with chronic viral hepatitis C was higher (score=1.78±0.44) than that in patients with chronic viral hepatitis B (score=1.4±0.55), but the difference was not significant ($p>0.05$). The average intensity score of LCN2 staining in the liver was 1.6 among all the specimens of chronic viral hepatitis. The most striking finding in the liver of both chronic viral hepatitis B and C cases was that the expression of LCN2 was significantly augmented with the presence of fatty infiltration (score:1.77±0.44) compared with the cases without fat in the liver (1.17±0.41) ($p<0.05$).

LCN2 staining was highest (score=2.93±0.27) in the liver specimens from HCC cases. The tissues from LMA also had a high score (2.33±0.87); however, the difference between these neoplastic tissues did not reach significance ($p>0.05$). On the other hand, this strong expression of LCN2 in the neoplastic liver tissues was significantly higher only in HCC cases than in CHB and CHC cases ($p<0.001$ for both). These results are summarized in Tables 1 and 2.

DISCUSSION

Lipocalin-2 (LPC2) is found in various normal and pathological human tissues, and its expression is induced in various cells under harmful conditions, including cancer, toxicity, infection, and inflammation (6,17). In the present study, we found that LCN2 is expressed weakly in the hepatocytes in the periportal and central areas of the normal liver tissue; however, neutrophils in the portal areas were stained strongly as was expected.

Lipocalin-2 (LPC2) has been reported to have a modulatory function in the inflammatory response and was also found to be significantly upregulated during infections, including viral infections (9). The expression of LCN2 in the liver of chronic viral hepatitis specimens has not been well demonstrated earlier. In the present study, the expression of LCN2 in the liver showed an increased trend in either CHB or CHC cases compared with the normal liver. Although the expression of LCN2 in the liver of CHC cases was found to be more intense than that in CHB cases, the difference was not significant.

Lipocalin-2 (LPC2) is overexpressed in the hepatocytes of experimental liver fibrosis (18,19). Similarly, urinary LCN2 levels were found to be increased in CHC patients (10), which has been suggested to reflect the severity of fibrosis in these patients. However, in the present study, the areas of fibrosis in the liver were not positive for LCN2 both in the chronic viral hepatitis B and in the chronic viral hepatitis C cases. In a very recent report from experimental liver injury models, it was reported that increased serum LCN2 levels are associated with liver injury; however, these increased levels do not necessarily correlate with the degree of actual fibrosis (20). Altogether, considering our results, it appears that LCN2 is not a direct indicator of liver fibrosis but rather a marker of inflammatory liver damage and homeostasis.

Fatty liver is commonly encountered in clinical practice. It is not only emerging as a sole entity but also accompanies other chronic liver diseases, including chronic viral hepatitis. We observed fatty infiltration in a considerable proportion (65%) of chronic viral hepatitis tissues. The striking and a hitherto unestablished finding of the pres-

ent study is the significant increase in LCN2 expression in the liver with the presence of fatty infiltration in chronic viral hepatitis cases. The stronger but insignificant higher expression of LCN2 in CHC cases than in CHB cases may also be due to more frequent presence of fatty infiltration in CHC than in CHB cases. There is no report in the literature that has investigated the relationship between LCN2 expression and steatohepatitis, and further studies are warranted to support and extend this novel finding.

Hepatocarcinogenesis is a long-term multistep process involving multiple risk factors and different genetic alterations that ultimately lead to malignant transformation of the hepatocytes (21). The study of tissue markers should be able to distinguish early HCC from other entities. LCN2 has been identified as a gene that is highly upregulated in HCC tissues (13,14,22). We also found an increased expression of LCN2 in HCC tissues consistent with the previous reports, and it was significantly higher than that in both CHB and CHC cases. However, LCN2 was expressed similarly in HCC and adenocarcinoma metastasis to liver in terms of staining properties and intensity. These findings clearly indicate that the expression of LCN2, which is found to be increased in chronic inflammation of the liver with viruses, appears to enhance during the development of neoplastic process, with the highest expression in HCC cases compared to other diseased conditions. However, this finding does not have a favorable role in discriminating HCC from liver metastasis of adenomatous tumors. One possible explanation for this finding is that LCN2 expression is not a part of the neoplastic process itself but rather a phenotype induced in neoplastic cells by an accompanying inflammatory reaction (23,24). The metastasis of neuorendocrine tumors to the liver was stained weakly with LCN2, which was comparable with that in the normal liver. There is no study in the literature reporting the expression of LCN2 in liver metastasis of neuorendocrine tumors, which is also a new finding in the present study.

Several mechanisms have been attributed to the expression of LCN2 in the liver. LCN2 was shown to increase in the hepatic inflammatory response (25). It is possible that LCN2 protects cells from toxic stress through an interaction with certain proteins, thereby modulating the functions of those proteins and has an ability to act as a scavenger for unwanted endogenous or exogenous compounds. In addition to the underlying chronic inflammation, the increase of LCN2 expression in the presence of toxic stimuli might also have an additive effect in the upregulation of LCN2 during the process of neoplastic liver conditions (23,24).

Despite the abovementioned findings, there are some limitations in our study. The expression of LCN2 in the liver tissue in diverse conditions, including malignancy, was analyzed only by immunochemistry. Using methods such as PCR and Western blotting would not only confirm our results but would also lead to more rigorous conclusions. On the other hand, the sample size of the present study was small. However, the study has a strong power of >0.80 calculated using power analysis.

In conclusion, the expression of LCN2 in the liver increases during chronic viral hepatitis. Importantly, LCN2 is not found in fibrosis areas, indicating that it is not a direct indicator of liver fibrosis. Nevertheless, the most striking and novel finding of the present study is the increased expression of LCN2 in the liver in chronic viral hepatitis with the presence of fatty infiltration. This result warrants further studies to investigate the relationship between LCN2 and other conditions with fatty liver such as steatohepatitis. The expression of LCN2 in the liver was strongest during neoplasia, but it was similar in both HCC and adenocarcinoma metastasis to liver and does not aid in discriminating these two neoplastic entities. These findings indicate that the expression of LCN2 in the liver increases with chronic inflammation and fatty infiltration and the overexpression encountered during neoplasia is not inherent in the neoplastic cell, but rather a result of accompanying inflammatory reactions. Further studies using methods such as PCR and Western blotting are warranted to reach more rigorous conclusions.

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