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CLINICAL RESEARCH / KLİNİK ARAŞTIRMA

Histopathological and Immunohistochemical Parameters (TGF-β1, FSP1-S100A4, α-Straight Muscle Actin, Collagen Type 1 and E-Cadherin) Conclusion of the Fibrogenesis Process in Chronic Liver Disease

Kronik Karaciğer Hastalığında Fibrogenez Sürecinin Histopatolojik ve İmmünohistokimyasal Parametreler (TGF-β1, FSP1-S100A4, α-Straight Kas Aktin, Kollajen Tip 1 ve E-Kadherin) ile Değerlendirilmesi

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Abstract

Objective: In any toxic or inflammatory damage to the liver, the function of transforming growth factor-beta (TGF-ß), a fibrogenic cytokine, is to secrete collagen type 1 through hepatic stellate cells (HSCs) and to ensure the restructuring of extracellular matrix. Recently, it has been thought that not only HSCs but also epithelial to mesenchymal transition (EMT)-showing cells secrete extracellular matrix proteins in the pathogenesis of fibrosis. The aim of this study was to show the relationship of activated HSCs and TGF-ß, with the changing stages of fibrosis and the presence of epithelial mesenchymal-transforming cells.

Methods: A total of 70 liver needle biopsy materials, including chronic viral hepatitis with different fibrosis scores evaluated in our department, and 20 control biopsies have been submitted to immunohistochemical (IHC) staining with IHC markers TGF-ß1, FSP1/S100A4, alpha (α)-smooth muscle actin, collagen-type 1, and E-cadherin.

Results: With an increased degree of fibrosis and inflammation TGF-β1, FSP1/S100A4, α-smooth muscle actin and E-cadherin and collagen type 1 expression have increased. This finding suggests that these molecules play a role in the process of fibrogenesis. A positive correlation was found between the expression of TGF-β1, α-smooth muscle actin, and E-cadherin, and collagen-type 1 relative to hepatitis B in chronic hepatitis cases, especially those associated with hepatitis C. It has been concluded that FSP1/S100A4 is not specific because it also marks other inflammatory cells besides fibroblasts. Hepatocytes, which show epithelial and mesenchymal transition, were detected in the liver by the dual IHC staining method.

Conclusion: Our findings support the idea that a mechanism of fibrogenesis in the liver occurs due to an increase in α-smooth muscle actin via fibrogenic-acting TGF-ß. Additionally, although only one study on IHC is not enough, the appearance of hepatocytes showing EMT suggests that this mechanism also contributes to the development of fibrosis.

Keywords: Chronic liver disease, fibrogenesis, immunohistochemical parameters, TGF-β1, FSP1-S100A4, α-straight muscle actin, collagen type 1 and E-cadherine

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Öz

Amaç: Karaciğere verilen herhangi bir toksik veya enflamatuvar hasarda, fibrojenik bir sitokin olan dönüştürücü büyüme faktörü-beta'nın (TGF-ß) işlevi, hepatik stellat hücreleri (HSC) yoluyla kollajen tip 1 salgılamak ve hücre dışı matrisin yeniden yapılandırılmasını sağlamaktır. Son yıllarda fibrozis patogenezinde sadece HSC'lerin değil, aynı zamanda epitelden mezenkimal geçişe (EMT) geçiş gösteren hücrelerin hücre dışı matriks proteinleri salgıladığı düşünülmektedir. Bu çalışmanın amacı, aktive olmuş HSC'ler ile TGF-ß'nin fibrozisin değişen evreleri ve epitelyal mezenkimal transforme edici hücrelerin varlığı ile ilişkisini göstermektir.

Yöntem: Bölümümüzde değerlendirilen farklı fibroz skorlarına sahip kronik viral hepatit olmak üzere toplam 70 karaciğer iğne biyopsisi materyali ve 20 kontrol biyopsisinin tümü immünohistokimyasal (IHC) belirteçler TGF-ß1, FSP1/S100A4, alfa (α)-düz kas aktin, kollajen tip 1 ve E-kadherin ile IHC boyama yapılmış ve incelenmiştir.

Bulgular: Artmış fibrozis ve enflamasyon derecesi ile TGF-β1, FSP1/S100A4, α-düz kas aktin ve E-kadherin ve kollajen tip 1 ekspresyonları artmıştır. Bu bulgu, bu moleküllerin fibrogenez sürecinde rol oynadığını göstermektedir. Kronik hepatit vakalarında, özellikle hepatit C ile ilişkili olanlarda, hepatit B'ye göre TGF-β1, α-düz kas aktin ve E-kadherin ve kollajen tip 1 ekspresyonu arasında pozitif korelasyon bulundu. FSP1/S100A4'ün spesifik olmadığı, çünkü fibroblastların yanı sıra diğer enflamatuvar hücreleri de işaretlediği sonucuna varılmıştır. Çift IHC boyama yöntemi ile karaciğerde EMT gösteren hepatositler saptandı.

Sonuç: Bulgularımız, karaciğerdeki fibrogenez mekanizmalarından birinin, fibrojenik etkili TGF-ß yoluyla α-düz kas aktinindeki artışa bağlı olarak ortaya çıktığı fikrini desteklemektedir.

Anahtar Kelimeler: Kronik karaciğer hastalığı, fibrogenez, immünohistokimyasal parametreler, TGF-β1, FSP1-S100A4, α-straight kas aktin, kollajen tip 1 ve E-kadherin

Introduction

Many factors that cause a chronic inflammatory response in the liver lead to fibrosis, which is a complex process that protects the organ from damage. The major fibrogenic cells that initiate fibrosis in the liver are hepatic stellate cells[1]. These cells with myofibroblastic activity cause contraction in the sinusoids and fill the disse space with collagen that they themselves produce. This, in turn, causes fibrosis. But fibrogenesis can occur in ways that do not involve hepatic stellate cells (HSCs) as well. Another hypothesis considered recently is that hepatocytes or cholangiocytes also undergo epithelial mesenchymal changes and acquire properties that can secrete extracellular matrix proteins. Epithelial-mesenchymal transformation in these cells indicates the production of *de-novo* expression of alpha (α)-smooth muscle actin, fibroblast specific protein-S100A4 and vimentin (basal membrane components, fibronectin and type 1/3 collagen molecules)[2-4]. Our study investigated the presence of epithelial and mesenchymal immune-staining cells in the area of epithelial mesenchymal transformation in the periportal area in liver biopsy materials.

Materials and Methods

In our study, 70 cases from the archive of the Department of Pathology of Manisa Celal Bayar University that were diagnosed with chronic hepatitis by liver biopsy between 1999 and 2012 were evaluated. The degree of fibrosis varies between 0 and 6 according to the Ishak method of scoring[5]. The cases were gathered in such a way that there were 10 pieces for each stage of fibrosis. It is also worth noting that these were all patients with viral hepatitis, including hepatitis B (HBV) or hepatitis C (HCV). Other causes of chronic hepatitis in patients (drug or alcohol use, toxic causes, metabolic disease, biliary tract diseases, etc.) were excluded. Additionally, the normal areas of liver tissue in the biopsies of 20 patients who had been excised for reasons such as tumor and hydatid cysts were evaluated as a control group. Immunohistochemical staining of α-smooth muscle actin, with FSP-S10A4, and transforming growth factor-beta (TGF-β) as a fibrogenic cytokine was performed to evaluate the myofibroblastic (HSCs ratio) activity in all cases. Additionally, E-cadherin antibodies stained membranously with cytoplasmic-stained collagen type 1 were applied by dual immunohistochemistry method and the cells expressing these two primary antibodies at the same time were evaluated.

α-smooth muscle actin, FSP-S10A4 and TGF-β immunohistochemical dyes were applied to the sections obtained from formalin-detected, paraffin-embedded tissues. Of these immunohistochemical determinants, all but TGF-β1 were stained with a fully automatic immunohistochemical staining device (Ventana). Anti-TGF-β1 antibody was applied after incubation at +4 degrees for 12 h by the hand staining method.

Immunohistochemical dyes of collagen type 1 and E-cadherin were studied with a fully automatic dyeing device using the dual dyeing method. Membranously stained E-cadherin was used as the first antibody, and collagen type 1, which is a cytoplasmic determinant, was used as the second antibody.

Evaluation of Immunohistochemical Results

All evaluations were performed under a standard light microscope, α-smooth muscle actin, in the control group and normally, stains the vascular smooth muscles in the portal areas of the liver. It did not exhibit any staining within the parenchyma. HSCs, on the other hand, are located on the edge of sinusoids and are not visible in the light microscopic evaluation. However, when activated, they are stained with α-smooth muscle actin. In our study group, the expression of α-smooth muscle actin was evaluated in the fibrous septa and periportal parenchymal areas in patients with fibrosis stage 0-1-2-3-4-5-6 (Figure 1). The rate and intensity of activated HSC staining in fibrous septa and cirrhotic nodules were also examined in cirrhosis cases. The evaluation of the staining score was performed as follows:

- **0 (negative):** No staining or staining of less than 3% of the area,
- **+1 (light):** 3-33% of the area is painted at a magnification of 10,
- **+2 (medium):** 34-66% of the area is painted at a magnification of 10,
- **+3 (severe):** staining of more than 66% of the area at a magnification of 10.

TGF-ß exhibits staining in hepatocytes and cholangiocytes at various stages of cirrhosis of the liver. When evaluating the expression of TGF-ß in our cases, the staining rate and density of hepatocytes in the parenchyma were examined, especially in the periportal areas. Granular staining of the cytoplasm of hepatocytes and cholangiocytes was evaluated as positive. The evaluation of TGF-ß staining score was performed in the same way as the staining scoring of α-smooth muscle actin (Figure 2).

In our cases, FSP-S100A4 was evaluated with fibroblasts in and around the portal areas and Kupffer cells in the sinusoids. Since FSP-S100A4 is also expressed by lymphocytes[6-8], its distinction from inflammatory cells was made by looking at the shape and placement of the cell. Round-shaped cells were evaluated as lymphocytes, and spindle-shaped ones were evaluated as fibroblasts. The density evaluation of fibroblasts stained with FSP-S100A4 was performed in the same manner as the staining score evaluation of TGF-ß and α-smooth muscle actin.

In hepatocytes using the dual immunohistochemistry method, collagen type 1 was stained in red cytoplasmically, while the brown of E-cadherin demonstrated membranous staining. Hepatocytes that simultaneously underwent cytoplasmic-red and brown-membranous staining around the fibrous bands and in the parenchyma [undergoing epithelial mesenchymal transition (EMT)], especially in the periportal areas, were evaluated. Brown hepatocytes with red colored collagen globules that were not on the cell membrane, and were clearly selected to be in the cytoplasm, were interpreted as positive. The density and intensity of staining were evaluated as follows:

- No staining=0
- Periportal-periceptal staining light=1
- Periportal-periceptal staining intense=2 (Figure 3).

All bile ducts in the portal areas were also examined. While only brown membranous staining with E-cadherin was observed in cholangiocytes, there was no evidence of cholangiocytes exhibiting both staining at the same time.

Statistical Analysis

The statistical analysis of the results was performed using version 15.0 of the Statistical Package for Social Sciences program running on a personal computer and Pearson and Spearman correlation analysis to investigate the correlation. When the results were analyzed by statistical methods, $p<0.05$ was considered significant.

Results

Of the cases included in the study, 42 (60%) were male and 28 (40%) were female. The ages of the patients ranged from 11 to 77 years, with the average age being 42.1 years. There was no significant relationship between age and sex. A total of 54 of the cases had HBV, and 16 had HCV-positive viral serology; 61 of the biopsies were obtained from thick needle biopsy, 9 were obtained from wedge biopsy. The control group consisted of 20 patients aged between 1 and 52 years. The patients must have had negative viral serological findings, undergone biopsy for non-hepatitis reasons and had a histologically "usual" appearance. Of the control group, 14 had thick needle biopsies and 6 received wedge biopsies.

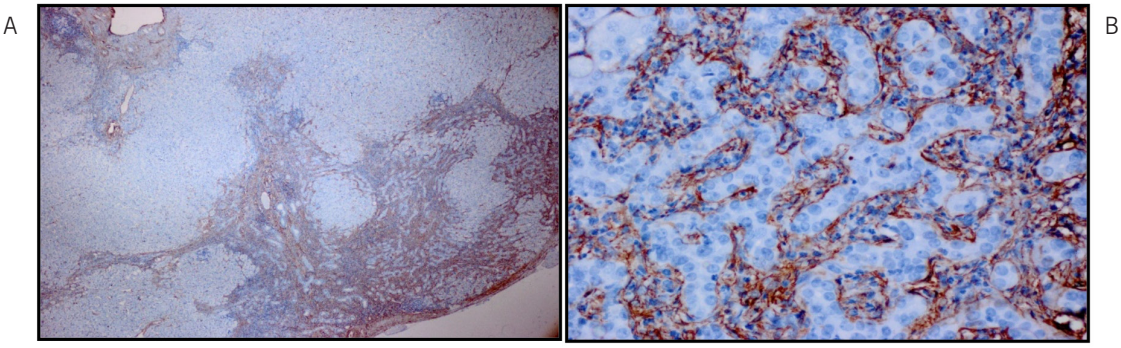

Figure 1. a, b) In wedge biopsy (x4-x40), alpha smooth November actin and areas in the cirrhosis liver (fibrosis score 6) were evaluated as severe intensity

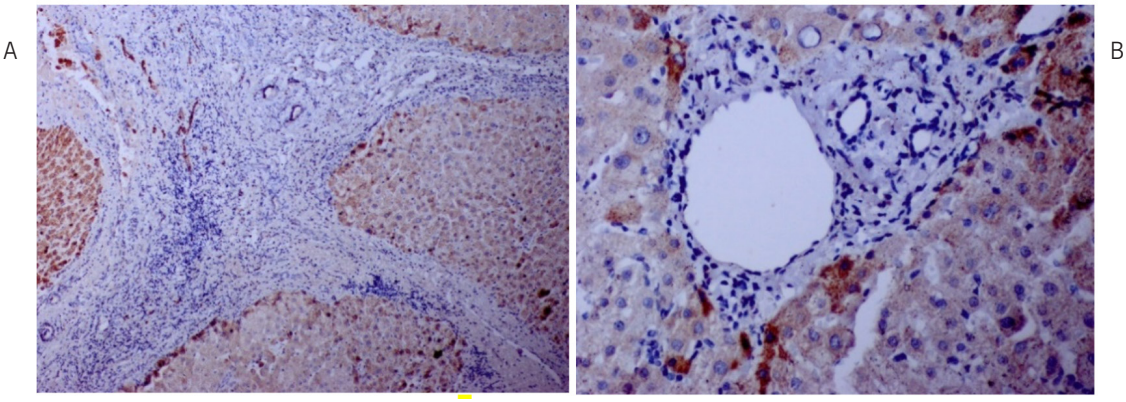

Figure 2. a, b) In wedge biopsy, TGF-ß staining (x20-x40) is concentrated in hepatocytes, especially in the periportal regions

TGF-ß: Transforming growth factor-beta

Immunohistochemical Results of the Cases

No staining was detected in 22 (31%) cases with smooth muscle actin. The cases who were not stained were included in the group fibrosis stages 0, 1, and 2. Severe (3+) staining was observed in 22 (31%) cases. The 3± stained patients included patients from the group with fibrosis stages 3, 4 and 5 and the entire group with stage 6. The staining in activated HSC cytoplasms, which are located in the sinusoids in the parenchyma starting from the periportal areas and extending to the periceptal region, was considered positive. In the control group, no staining was observed in the parenchyma, except for the vascular smooth muscle in the portal area. There was a positive correlation between the stage of fibrosis and the intensity of expression of smooth muscle actin in HSCs. This was also statistically significant (p=0.01).

No staining was observed in 20 (28%) cases TGF-ß. The cases without TGF-ß expression were included in the group with

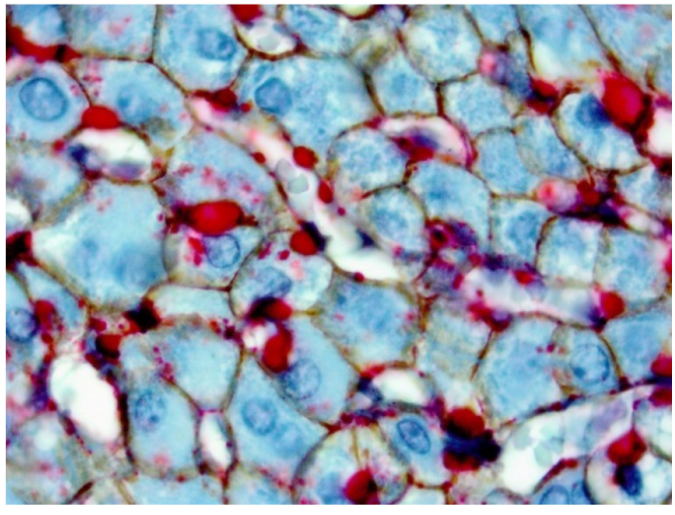

Figure 3. Hepatocytes (x100-x100), which show a double staining of e-cadherin-collagen, which is evaluated as slightly and severely concentrated on the periportal areas during a thick needle biopsy

fibrosis stages 0 and 1. Severe staining was observed in 17 (24%) cases. All 3+ cases were in the group with fibrosis stages 5 and 6. In our cases, cytoplasmic, granular staining of hepatocytes was considered positive. The intensity of staining was increased in the entire parenchyma, especially in hepatocytes in the periportal areas. TGF-ß expression was also observed in bile ducts, but there was no relationship between this expression in cholangiocytes and the level and intensity of fibrosis. As the fibrosis stage increased, TGF-ß expression also increased. Accordingly, a statistically significant positive correlation was found between the stage of fibrosis and the expression of TGF-ß in hepatocytes (p=0.01). In the control group, staining with TGF-ß was observed only in the normal bile ducts, while no staining was observed in hepatocytes either in periportal or pericеptal areas.

No staining was detected in 4 (6%) cases with FSP-S100A4, and all of these cases that were not stained were in the group with fibrosis stage "0". Severe staining was detected in 32 (46%) cases. These cases included part of the group with stage 4 fibrosis and the entire group with stages 5 and 6 fibrosis. FSP-S100A4 was also observed to stain lymphocytes, plasma cells and Kupffer cells of macrophage origin. For this reason, in the evaluation of this antibody that is not specific to fibroblasts, the spindle-shaped-cell criterion was taken to decide on fibroblastic activation. Round-nucleated cells in these areas were considered inflammatory cells, and spindle-shaped cells located in the periportal area were considered fibroblasts. Since the portal and periportal areas were evaluated for FSP-S100A4 staining, Kupffer cells with sinusoidal placement created fewer problems. There was also a statistically confirmed positive correlation between the stage of fibrosis and FSP-S100A4 (p=0.01). In 5 cases in the control group, a few occurrences of staining with FSP-S100A4 were detected in lymphocytes in the portal area, as well as spindle-shaped fibroblasts on the periphery of the portal area and Kupffer cells in the sinusoids. These cases were evaluated as 1+ FSP-S100A4 positivity and interpreted as an indicator of fibroblastic activation.

Staining characteristics of dual antibodies (E-cadherin-collagen): No staining was detected in 38% (54%) of cases, while the number of those stained with 1+ was 27 (38.9%) and those with 2+ staining was 5 (7.1%).

Discussion

The hepatic fibrosis cascade develops in several stages: activation of HSCs and Kupffer cells, the migration of HSCs, proliferation, synthesis and storage of extracellular matrix, rearrangement of scar tissue, fibrosis contraction, and eventual apoptosis of HSCs. During this process, the proliferation and differentiation of HSCs is proportional to the development of hepatic fibrosis. Hepatic stellate cells are cells that play a major role in the excessive storage of connective tissue components. It has been shown in many previous studies that activated HSCs express α-smooth muscle actin[9,10]. It is believed that TGF-ß, the main fibrogenic cytokine, plays a role in the formation of these events. However, when liver damage develops, the type 1 collagen content also increases in response. The relationship between the density and activation of stellate cells and fibrosis has been investigated in various liver diseases.

Akpolat et al.[11] have evaluated the expression of α-smooth muscle actin by immunohistochemical methods in liver biopsy samples of patients with chronic HBV and cirrhosis and compared the actin levels with the fibrosis stage. They found that α-smooth muscle actin expression increased as the fibrosis stage progressed, and this expression was significantly clear, especially in the periportal, pericentral and pericinusoidal areas. Additionally, they also detected α-smooth muscle actin expression in a few cases that had not yet developed fibrosis and determined that these were also cases with particularly pronounced necro-inflammatory activity. Therefore, because of the study, they stated that the indication of periportal fibrosis only, in patients with chronic HBV may not be sufficient to assess the stage of fibrosis, especially in the early stages, α-smooth muscle actin expression will help detect fibrosis, and so, it may be a harbinger of fibrosis development.

Dooley et al.[12] on the other hand, have noted that not only α-smooth muscle actin but also collagen type 1 and TGF-β expression increased in parallel with inflammation-activated and proliferating HSCs. In this experimental study, they showed that anti-TGF-β therapy stops fibrotic development in the liver and reduces fibrogenesis. Chu et al.[13] also showed significant activation of HSC in chronic HBV and chronic HCV (which occurs more commonly) in a way that correlates with the degree of necroinflammation and the stage of fibrosis.

Tomanovic et al.[14] have studied the activated HSC density by examining alpha-smooth muscle actin through the immunohistochemical method in patients with chronic HCV. They did not find a significant relationship with the degree of necro-inflammatory activity when monitoring a positive correlation between activated HSC density and fibrosis. Additionally, they showed that activated HSCs were significantly present in the portal area and fibrous septa.

As can be seen from these studies, α-smooth muscle actin expression in liver damage shows an increase parallel to HSC activation. Based on these findings, α-smooth muscle actin expression, which we can consider as a reliable predictor of HSC activation, also shows a significant correlation with fibrosis stage in our study ($p<0.05$). Additionally, significantly more α-smooth muscle actin expression was detected in our cirrhotic patients compared to other stages of fibrosis. This finding suggests that a more intense activation of HSC occurs at the stage of cirrhosis, rather than in the development of fibrosis. The absence of α-smooth muscle actin staining in the periportal zone and parenchyma in any case in our control group supports the that damage to the liver and the resulting inflammation trigger HSC activation. A positive correlation between the degree of necro-inflammatory activity, fibrosis and α-smooth muscle actin expression was also observed in our study. However, no cases with fibrosis stage "0" showing actin expression were detected in our study. This finding contradicts the view put forward by Akpolat et al.[11] that "actin is a predictor of early fibrosis". When we looked at the relationship with viral serology, it was observed that α-smooth muscle actin expression was higher in our HCV-positive patient group than in HBV-positive cases. All these findings, which coincide with the literature, suggest that HSC activation is the main-guiding step not only in the development of fibrogenesis in chronic hepatitis, but also from the point where cell damage begins.

Additionally, in our study, α-smooth muscle actin, TGF-β expression and E-cadherin and type 1 collagen dual expression was significantly higher at the stage of fibrosis in HCV-positive patients, than in HBV. This has led to the idea that HCV is more effective than HBV in the progression to fibrosis and, as a result, cirrhosis.

It has been reported that TGF-ß is expressed in the liver only in case of damage, it is not expressed in a normal liver[15,16]. In parallel, TGF-ß expression was not detected in hepatocytes in the control group in our study. This finding supports the idea that there is no expression of TGF-ß without primary damage to the liver. In chronic hepatitis cases, TGF-ß expression increases in accordance with the fibrosis score. A statistically positive correlation was found between TGF-ß expression and both necro-inflammatory activity and fibrosis stage ($p<0.05$). When examined morphologically in detail, it has been observed that this expression is concentrated in hepatocytes in the periportal region. Additionally, when hepatitis and cirrhosis cases are compared in terms of TGF-ß expression, it is noted that TGF-ß expression is significantly higher in the cirrhosis group. Again, in our study, it was found that TGF-ß expression was higher in HCV-positive patients than in HBV. This finding leads to the idea that HCV further stimulates inflammation and the resulting fibrosis. In addition to hepatocytes, TGF-ß is expressed by bile ducts[17,18], but in our cases with a different fibrosis score, no relationship is seen between bile ducts and TGF-ß expression.

In our study, there was no statistically significant relationship between age and gender and the prevalence and intensity of TGF-ß expression. FSP-S100A4, the antibody that we had the most evaluation problems with, showed intense staining in lymphocytes, macrophages, fibroblasts in the periportal area, and Kupffer cells in the parenchyma. This nonspecific staining has shown us that FSP-S100A4, which was originally suggested to be a predictor of myofibroblastic activity, is actually a predictor of cells of macrophage/fibroblast origin. Despite this, the problem in evaluating FSP-S100A4 has been overcome by looking at the localization and shape of the cells. Although this problem worsened the objective assessment, a positive correlation was observed between the staining intensity of FSP-S100A4 and the fibrosis level and histological activity index ($p<0.05$). However, there was no significant difference in terms of the type of viral hepatitis. The interpretation of mild FSP-S100A4 expression observed in 5 cases in the control group is that "although close to normal, there may be a slight level of fibroblastic activation due to any damage in these cases." In our study, it was concluded that FSP-S100A4 is a predictor of necro-inflammatory activity rather than myofibroblastic activity due to the width of the stained cell population and problems in the evaluation. According to Österreicher et al.[19] of human liver tissue and stellate cell culture, it was found that FSPS1A4, similar to our results, is not a specific determinant for myofibroblastic cells.

The cells that cause fibrosis in the liver are activated HSCs and fibroblasts with myofibroblastic activity. However, in recent studies, it is thought that the EMT mechanism, which leads to fibrosis other than these two cells, also leads to fibrosis. EMT, which also plays a role in embryogenesis, tumor invasion and metastasis, is the process by which a cell of the epithelial nature acquires a mesenchymal property over time. It is thought that the cells that cause EMT in the liver are hepatocytes or cholangiocytes[20-23].

In our study, hepatocytes and cholangiocytes expressing membranous-stained E-cadherin and cytoplasmic-stained collagen type 1 at the same time were investigated by the dual-immunohistochemical staining method. It has

been observed that hepatocytes with dual staining are concentrated in the periportal and periceptal regions. The presence of a positive correlation between fibrosis and histological activity and the density of cells exhibiting dual staining supports the idea that this transformation occurs in the case of hepatic damage. However, none of these cells showed loss of E-cadherin, which is an epithelial marker. From a theoretical perspective, at the stage of EMT formation, the hepatocyte first loses its adhesion molecules, removes its epithelial connections and is removed from the hepatocyte cord and acquires mesenchymal properties. According to our study, two possible explanations for this deviation during the development of events are given. The first is the possibility that collagen expression begins before epithelial features disappear. In our microscopic observations, the fact that we see collagen globules as few and small in the hepatocyte cytoplasm supports this idea. The second possibility is that although immunohistochemical staining methods have been standardized, more sophisticated approaches and molecular studies must evaluate molecular expressions.

In our evaluation of EMT, another cell with dual staining, cholangiocytes, was investigated, but cholangiocytes expressing both immunohistochemical markers together were not detected.

In our study, considering all of this information and recent research findings, expression of TGF-ß-1, α-smooth muscle actin, FSP-S100A4, collagen type 1 and molecules of e-cadherin, which are the most mentioned in the process of pathogenesis and play the most important role in it, in liver tissue with chronic active hepatitis, cirrhosis and normal liver tissue were investigated. Answers to questions such as "Is there a change in the expression of these molecules in liver tissue that exhibit different stages of fibrosis?" and "If there is, can this guide us in patient follow-up and treatment?" have been sought. Our findings support the that a mechanism of fibrogenesis in the liver occurs due to an increase in α-smooth muscle actin via fibrogenic-acting TGF-ß[24]. Additionally, although only a single study regarding immunohistochemical staining is not enough, the appearance of hepatocytes showing EMT suggests that this mechanism also contributes to the development of fibrosis.

Study Limitations

No restrictions were encountered during the planning or construction of this study.

Conclusion

As far as we can observe, these molecules have been studied separately in the literature, but there is no research conducted on human tissue that shows the relationship of all of them with each other. Currently, liver fibrosis can be prevented, and cirrhosis is also considered a reversible pathological process. New studies are focusing on understanding the steps of fibrogenesis and preventing this process from occurring.

Ethics

Ethics Committee Approval: The study was approved by the Manisa Celal Bayar University of Local Ethics Committee (protocol no: 204, date: 09.06.2011).

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.A., Design: T.K., S.A., Data Collection or Processing: T.K., A.R.K., A.İ., Analysis or Interpretation: T.K., A.R.K., A.İ., Literature Search: T.K., Writing: T.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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