

## Original Article

# Comparison of transforming growth factor- $\beta$ (TGF- $\beta$ ) expression between diabetes mellitus associated carpal tunnel syndrome and idiopathic carpal tunnel syndrome

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Received September 22, 2015; Accepted January 6, 2016; Epub February 15, 2016; Published February 29, 2016

**Abstract:** Background: Carpal Tunnel Syndrome (CTS) is a trap neuropathy which occurs when Nervous Medianus gets compressed in the carpal tunnel. Fibrosis of subsynovial connective tissue in etiopathogenesis of CTS occupies an important place. Systemic factors facilitate the formation of the syndrome. In this study, collagen structure and fibrosis of subsynovial connective tissue in patients with diabetic and idiopathic CTS cases were evaluated. Methods: Subsynovial connective tissue samples of 30 diabetes mellitus patients with CTS and 30 subsynovial connective tissue samples of idiopathic CTS cases were used in the study. Antibodies of Collagen Type I, Collagen Type II, Collagen Type III and Collagen Type VI were used to evaluate the collagen structure. Expression of Transforming Growth Factor- $\beta$  (TGF- $\beta$ ), an important molecule held responsible of non-inflammatory fibrosis, was evaluated by comparison in both patient groups. Results: Specific staining was not observed in subsynovial connective tissue with Collagen Type I and Collagen Type II. Collagen Type III and Collagen Type VI were positive in subsynovial connective tissue and a significant difference in staining intensity was not observed in diabetic and idiopathic CTS group ( $P > 0.01$ ). Expression of Collagen Type III was seen as more intense in the vessel wall in the diabetic CTS group. ( $P < 0.01$ ). It was observed that TGF- $\beta$  expression in the diabetic group was weaker than the idiopathic CTS group. ( $P < 0.01$ ). Conclusion: Collagen Type VI is seen as the major component of the subsynovial connective tissue. Decrease in TGF- $\beta$  expression in subsynovial connective tissue was observed in diabetic CTS cases similar to the healing model of wounds in diabetic patients. We believe that microvascular changes and metabolic factors are effective more than TGF- $\beta$  expression in subsynovial connective tissue changes in diabetic patients with CTS. As a result, diabetic patients with CTS consisting the majority of CTS cases must be evaluated as a separate group of patients while histomorphological changes in CTS are evaluated.

**Keywords:** Carpal tunnel syndrome, diabetes mellitus, subsynovial connective tissue, transforming growth factor- $\beta$

## Introduction

Carpal tunnel syndrome is a common peripheral neuropathy caused by pressure of the median nerve (N. medianus) in the carpal tunnel [1]. The narrowing of the tunnel or increase in its content results with CTS [2]. Subsynovial connective tissue, which is made of multi-layered collagen fibril filaments, prevents greasing during the movement of the tendons and protects the vascular structures in it [2].

Recent studies emphasize noninflammatory fibrosis and trap of the nerve caused by the fibrosis as etiopathogenesis of the CTS [3-6]. Transforming growth factor beta (TGF- $\beta$ ) is a profibrotic cytokin and responsible for the non-inflammatory fibrosis.

Recently, blockage of TGF- $\beta$  expression is shown to be a target for the therapy of non-inflammatory fibrosis developed in CTS since the expression of TGF- $\beta$  is found to be increased

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in the subsynovial connective tissue of the CTS with non-inflammatory fibrosis [3, 4]. Systemic or local causes, such as trauma or soft tissue tumors causing pressure on the pressure area can lead to CTS which is mostly idiopathic [7, 8]. However, in case series of Lewanska et al. In this series, obesity, thyroid dysfunction, hormone replacement therapy, oophorectomy and diabetes mellitus with a share of 12% are numerable as facilitating systemic factors of CTS [9].

Most of our operated CTS patients are diagnosed with diabetes mellitus.

Previous reports about CTS are mostly consisted of patients with idiopathic CTS. However, in the literature, there was no immunohistochemical study about the changes in the subsynovial connective tissue of the CTS patients with diabetes. Thus, present study aims to investigate the role of idiopathic subsynovial fibrosis and TGF- $\beta$  expression in CTS patients and also evaluate the efficacy of the blockage of TGF- $\beta$  expression in diabetic CTS.

### Material-method

Subsynovial connective tissue samples of 30 diabetes mellitus patients and 30 idiopathic CTS patients were evaluated retrospectively. Idiopathic CTS patients were operated between the years of 2011-2012.

An informed consent form was obtained from all of the patients. IRB approval was not necessary for our institution because of our study was a Level 3 retrospective study.

The stories of paresthesia along with median nerve trace and hand pain during the day or night were taken into consideration to confirm the diagnosis of CTS in patients.

In addition, to confirm CTS, the positive findings including sensibility and provocative tests during the physical examination were used.

In this study, the patients whose Hemoglobin A1c results are above 7 and with the diagnosis of diabetes mellitus at least 5 years before were included to study according to the results of blood biochemistry.

Electromyography (EMG) was performed to all patient that suspected CTS. The majority of

patients diagnosed as CTS according physical examination mentioned above and laboratory findings, had received splints, non-steroidal anti-inflammatory drugs, vitamin B12 treatment.

Despite this treatment, the patients were operated due to ongoing complaints. A single surgeon made the standard open carpal tunnel release procedure. In peripheral nerve surgery, the minimally invasive technique under local anesthesia was applied to patients. 1 cm vertical incision from the wrist bend to fit the imaginary line between the middle finger and ring finger on the palmar side of the hand was made.

A broader view of the proximal and distal directions was achieved by using the elasticity of the skin with the help of ecarteurs. The skin incision and subcutaneous incision were made respectively. The median nerve was exposed by cutting about 3-5 cm from proximal to distal with seeing the transverse carpal ligament colored bright pearl.

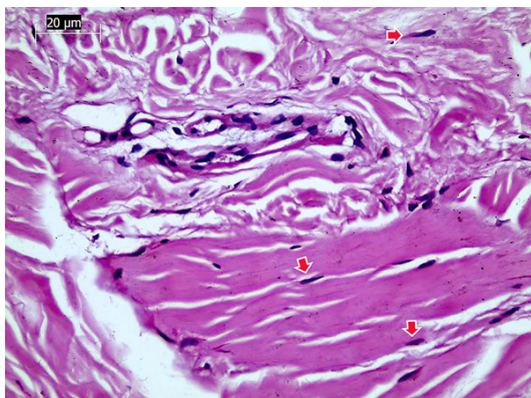
About 0.5 mm<sup>3</sup> subsynovial connective tissue was excised from each case. For decrease the hydrostatic pressure about 1 cc saline solution was given to perineurium. Thus the decompression of carpal tunnel was performed. The connective tissue samples within 10% buffered formalin were immediately sent to histopathology laboratory. 4 micron-thick sections were taken from the paraffin-embedded blocks prepared from the tissue samples. The standard hematoxylin-eosin staining procedure was performed at room temperature.

Immunohistochemical examinations with Collagen type I, Type II, Type III, Collagen Type VI antibodies to subsynovial connective tissue samples were made in both study groups to evaluate the structure of the subsynovial connective tissues. Expression of TGF- $\beta$  in diabetic and idiopathic CTS cases was evaluated.

Antibodies and clones used for immunohistochemical examination are as follows: Collagen I antibody (Bioss); Collagen II Antibody (Neomarkers clone 6B3); Collagen III Antibody (Bioss); Collagen VI Antibody (Bioss); TGF- $\beta$  antibody (santa-Cruz biotechnology-Clone TB21).

4 micron-thick sections were prepared for immunohistochemical examinations. Then de-

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**Figure 1.** Noninflammatory fibrosis, proliferating fibroblasts are observed (arrows) H&E  $\times 40$ .

paraffinization study was applied to the sections. The deparaffinization procedure was made with xylene in the oven at 60°C. Then, the examples were dehydrated using descending concentrations of ethanol. (Absolute alcohol-alcohol-96% to 90% alcohol-70% alcohol).

The preparations were cleaned under running tap water and distilled water. Then, in the antigen retrieval procedure, citrate buffer (pH 6) was applied to preparations for 5 minutes under pressure. After the primary antibody solution, the hydrogen peroxide, phosphate buffered saline (PBS-pH 7.4), ultra V block were applied respectively.

Preparations were kept in primary antibody for Collagen III, Collagen I, Collagen VI antibody and TGF- $\beta$  for 2 hours and for Collagen II antibody for an hour. PBS, amplifier, PBS, HRP Polymer, PBS, AEC chromogen, running tap water, hematoxylin dye, running tap water and distilled water were applied to slides respectively. Then slides were dried at room temperature and closed by lamellas.

The micro polymer system kit was used in all of the immunohistochemical studies. Negative control for immunohistochemistry was processed without the primary antibody.

The assessment of hematoxylin-eosin stained preparations and immunohistochemical studies and photo shooting was made by Olympus CX41 light microscope. Hemotoxylin and eosin stained sections were evaluated histomorphologically in both patient groups. 8 randomly selected fields were used for evaluation of

Collagen Type I, Type II, Type III and Type VI. (Magnification set for 20 $\times$ ).

*In all these areas, staining*

Intensity was classified as follows: Grade 0: No stained Grade 1: Mild stained; Grade 2: Moderately Stained; Grade 3: Intensely Stained.

Stromal elements in the subsynovial connective tissue and staining intensity in the vessel walls were evaluated separately for each of the 4 immunohistochemical markers. In evaluation of the immunohistochemical examination made by TGF- $\beta$  eight different areas selected at random were examined at  $\times 20$  magnification. The percentage of fibroblasts giving stromal positive reactions to was evaluated. Positively stained vessels in randomly selected eight objective to evaluate staining in the vessel wall [2]. Man-Whitney U test was utilized to compare the results of immunohistochemical examinations statistically in diabetic CTS and idiopathic CTS groups.  $P < 0.01$  was considered significant value in interpretation of statistical data.

### Results

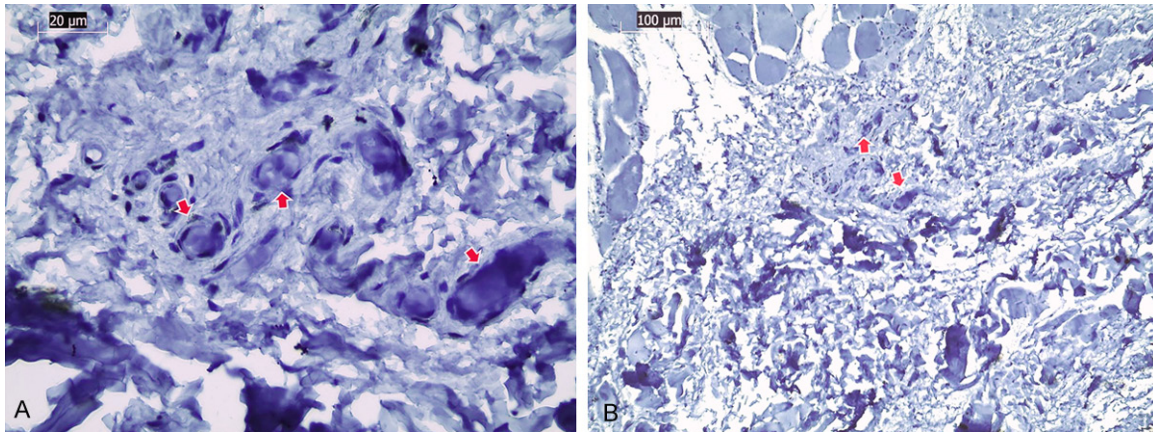
In both patient groups, in the routine pathologic examination prepared with hematoxylin-eosin stain, noninflammatory fibrosis was observed in the majority of tissue samples taken from patients (**Figure 1**).

Results of immunohistochemical examination: Specific staining was not seen for Collagen Type I and Type II in the subsynovial connective tissue in both patient groups. Positive staining was observed for Collagen Type I and Collagen Type II in a few vascular walls. Positive staining was observed for Collagen Type III and Collagen Type VI in the susynovial connective tissue and a few vascular walls.

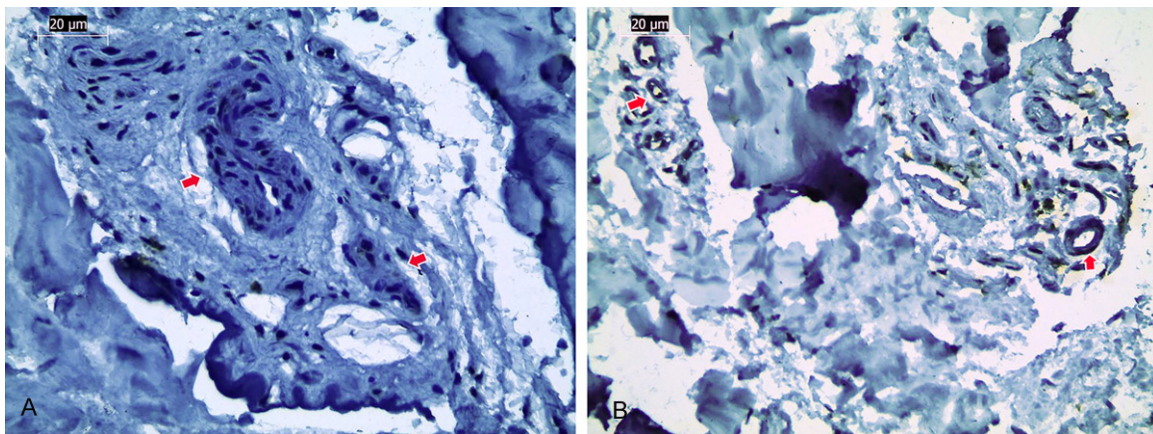
When stromal component staining intensity was evaluated for Collagen Type III and Collagen Type VI in the subsynovial connective tissue, a statistically significant difference was not observed between diabetic CTS and idiopathic CTS groups  $P > 0.01$ .

When staining intensity was evaluated for Collagen Type I, Collagen Type II, and Collagen Type VI in the vascular walls no significant dif-

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**Figure 2.** A. Positive staining is observed in a few vascular walls, idiopathic CTS (arrows), Collagene type I  $\times 40$ ; B. Positive staining is seen in a vascular walls, diabetic CTS (arrows), Collagene type I  $\times 10$ .



**Figure 3.** A. Vascular walls, positive staining is observed for collagene type II (arrows), idiopathic CTS, Collagene type II  $\times 40$ . B. Vascular walls, positive staining is observed for collagene type II (arrows), diabetic CTS, Collagene type II  $\times 40$ .

ference was found between the two groups in terms of staining intensity  $P > 0.01$ .

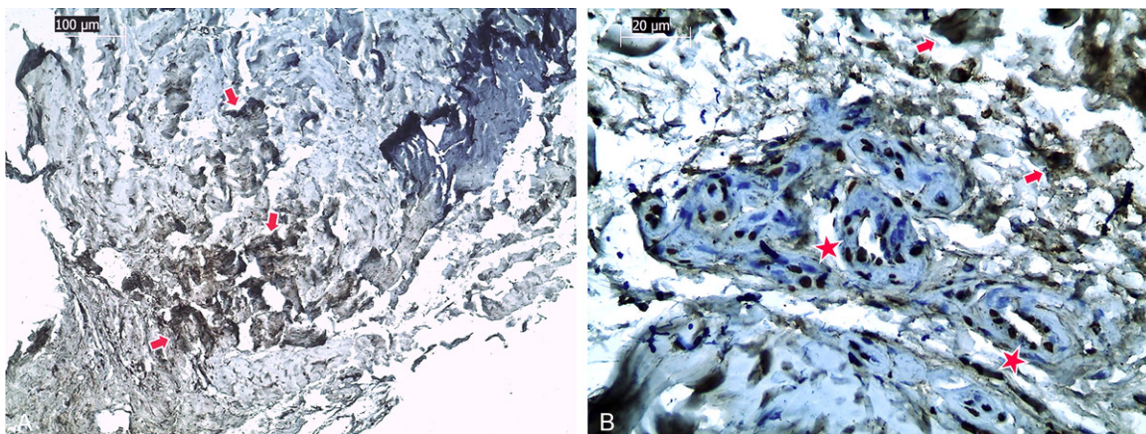
When staining intensity was evaluated for Collagen Type III in the vascular walls significantly intensive positive staining was observed in diabetic CTS group compared to idiopathic CTS group  $P < 0.01$  (Figures 2-5).

When rates of fibroblasts stained for TGF- $\beta$  in subsynovial connective tissue were evaluated fibroblasts rates giving positive reaction in idiopathic CTS group for TGF- $\beta$  changed from 25% to 1%. This rate was observed as 0% to 10% in diabetic CTS group. When the number of positive vessels for TGF- $\beta$  was counted the number of positively stained vessels in the idiopathic group changed from 1 to 25. This number was from 0 to 5 in the diabetic CTS group (Tables 1, 2).

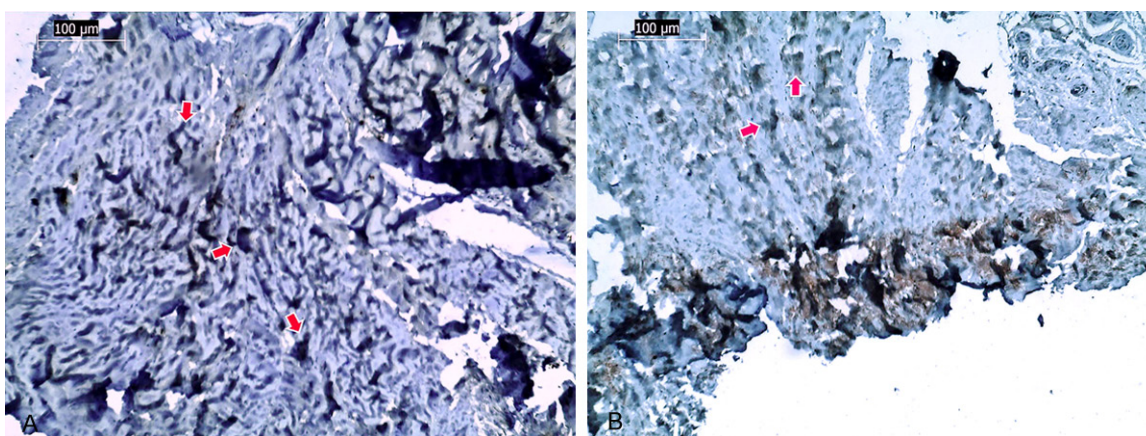
When the percentage of positively stained fibroblasts for TGF- $\beta$  in the stroma and vascular structures in the subsynovial connective tissue were evaluated significantly intensive positive staining was observed in idiopathic CTS group compared to diabetic CTS group in both components groups  $P < 0.01$  (Tables 3, 4). TGF- $\beta$  expression was observed to decrease in diabetic CTS group compared to idiopathic CTS group (Figure 6).

### Discussion

CTS occurs due to decrease in the tunnel itself or increase in its content [10, 11]. No significant difference between CTS positive and CTS negative groups was detected in biomechanical and morphometric behavior of the transverse carpal ligament [10]. Thus, CTS seems to



**Figure 4.** A. Positive staining is observed in the subsynovial connective tissue (arrows), idiopathic CTS Collagene Type III  $\times 10$ . B. Positive staining, in the subsynovial connective tissue (arrows) and intensive positive staining, in the vascular walls (stars), diabetic CTS Collagene type III  $\times 40$ .



**Figure 5.** A. Positive staining is observed in the subsynovial connective tissue (arrows), idiopathic CTS Collagene type VI  $\times 10$ . B. Positive staining is observed in the subsynovial connective tissue (arrows), diabetic CTS Collagene type VI  $\times 10$ .

be caused by the increase in volume of the inside of the carpal tunnel [10].

Recently, histopathological, immunohistochemical changes in subsynovial connective tissue became a current issue in the etiopathogenesis of CTS [2, 3, 10, 12]. Edema, non-inflammatory fibrosis, microvascular damage and thickening of the vessel wall in subsynovial connective tissue are observed in CTS [10, 13]. There are several immunohistochemical and electron microscopic studies investigate collagen organization in subsynovial connective tissue to assess fibrosis of the tissue more detailed.

In the immunohistochemical study conducted by Ettema et al., it is stated that, collagen type

I and II were absent in subsynovial connective tissue whereas collagen type VI is found to be the key component of the subsynovial connective tissue. In the same study, collagen type III is reported to be produced as an answer to injury of the subsynovial connective tissue and expressed higher in patient group in comparison with the study group [2].

Oh et al. found that collagen type I, III and VI in the subsynovial connective tissue both in the study group diagnosed with CTS and in the control group via electron microscope [13].

In the present study immunohistochemically, no expression of collagen type I and type II was found in the subsynovial connective tissue of the diabetic CTS and idiopathic CTS group.

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**Table 1.** Tgf-B expression in the fibroblasts

TGF-β expression the percentage of positively stained fibroblasts	Idiopathic Cts	Diabetic Cts
0-5%	12 patients	24 patients
6-10%	15 patients	6 patients
11-25%	3 patients	0 patient
Total	30 patients	30 patients

**Table 2.** Tgf-B expression in the vessels

TGF-β expression the number of positively stained vessels	Idiopathic Cts	Diabetic Cts
0-5	20 patients	30 patients
6-10	5 patients	0 patients
11-25	5 patients	0 patient
TOTAL	30 patients	30 patients

**Table 3.** TGF β Expression, percentage of fibroblasts, comparison of diabetic and idiopathic CTS, (P<0.01)

Groups	N	$\bar{x}_{sira}$	$\sum_{sira}$	U	Z	P
Diabetic	30	23.88	716.50	251.500	-2.963	.003
Idiopathic	30	37.12	1113.50			

**Table 4.** TGF β Expression, number of vessels, comparison of diabetic and idiopathic CTS, (P<0.01)

Groups	N	$\bar{x}_{sira}$	$\sum_{sira}$	U	Z	P
Diabetic	30	24.82	744.50	279.500	-2.665	.008
Idiopathic	30	36.18	1085.50			

Vessel walls were found to be stained with both of the collagen types. Yet, no difference was found in the manner of staining density between both groups.

Collagen type VI expression was found in the subsynovial tissue and in seldom vessel walls in the diabetic and idiopathic CTS and no difference was found between two patient groups in the manner of expression of collagen type VI. Collagen type VI is found to be the key component of the subsynovial connective tissue.

Both in diabetic and idiopathic CTS there is collagen type III expression in subsynovial connective tissue and in the vessel walls. Collagen type III expression is seen as response to injury. In the present study, collagen type III expression was found in both groups in subsynovial connective tissue and in the vessel walls. No difference was found between two patient

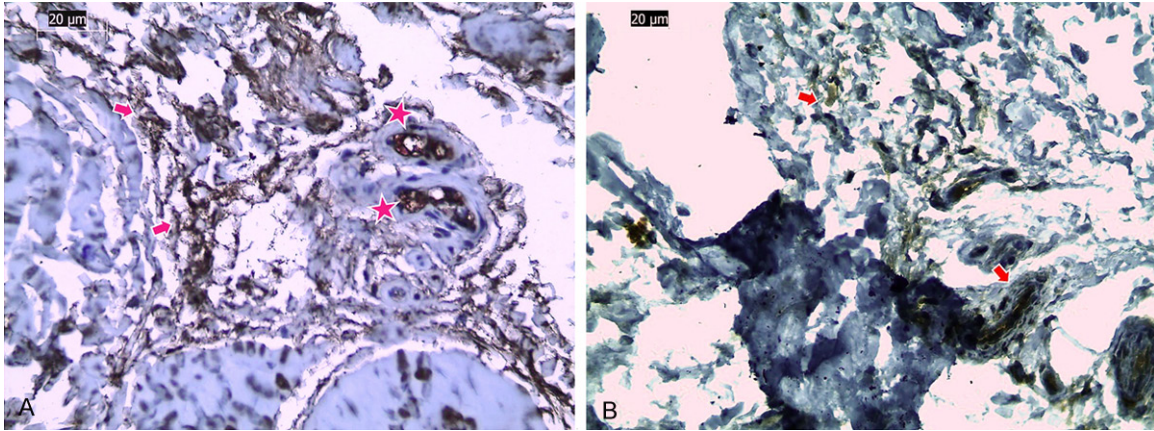
groups in the manner of expression of collagen type III in subsynovial connective tissue. However, expression of collagen type III was found to be statistically significantly higher in the diabetic CTS group. These findings endorse microvascular damage, thickening of the vessel wall in subsynovial connective tissue and neoangiogenesis concept in etiopathogenesis of diabetic CTS [7, 10, 13].

TGF-β, is a multifunctional growth factor well defined/related with fibrosis in many organs and systems [4]. TGF-β is responsible for the cell proliferation, adhesion, regulation and dysregulation of the migration. Besides, it has a role in production of the extracellular matrix, acute inflammatory fibrosis and remodeling process. However, recent studies showed that fibrosis can also be caused by oxidative stress in CTS, which is also TGF-β mediated. TGF-β is highly expressed whether the initial in-

flammation or an oxidative stress response is low in CTS [3].

In the study of Gingery et al, decrease in the fibrotic gene expression via fibrotic TGF-β receptor inhibition, as a specific anti-fibrotic treatment, in CTS is highlighted [4]. In the present study, TGF-β expression was found to be weaker in the subsynovial connective tissue and vessel wall in diabetic CTS group, which encompasses most of the CTS cases, compared to idiopathic CTS group.

Diabetes mellitus (DM) is a metabolic disease which is characterized with hyperglycemia. Poor wound healing is one of many complications of DM [14, 15]. Wound healing is a complex and multifactorial process including integration of inflammation, angiogenesis, formation of granulation tissue and collagen accumulation. Previous studies showed that wound healing is delayed in diabetic mice [16].



**Figure 6.** A. Intensive positive staining is observed in the subsynovial connective tissue (arrows) and vascular walls (stars), idiopathic CTS TGF- $\beta$   $\times 40$ . B. Mildly positive staining is observed in the subsynovial connective tissue (arrows), diabetic CTS TGF- $\beta$   $\times 40$ .

Wound healing defect in DM is caused by variable physiological factors [15]. These factors include phenotypical changes, decrease in growth factor production, collagen synthesis, fibroblast proliferation and migration [14].

TGF- $\beta$  seems to be the main mediator responsible from non-inflammatory fibrosis in DM. TGF- $\beta$  contributes to fibrosis in diabetic nephropathy as TGF- $\beta$  expression is found to be increased in kidney tissue in diabetic nephropathy [17-20]. TGF- $\beta$  also plays a role in acute inflammatory fibrosis and remodeling process. In the previous studies decrease in TGF- $\beta$  expression was reported in wound healing of diabetic cases [17, 21].

Our study is pioneer since there is no comprehensive study on TGF- $\beta$  expression in diabetic CTS. Decreased TGF- $\beta$  expression in subsynovial connective tissue is seen in diabetic CTS cases compared the idiopathic CTS cases. This condition may be interpreted like wound healing process. In the etiopathogenesis of diabetic patients with CTS seen very commonly, other factors than the expression of TGF $\beta$  should be considered to play a role.

### Conclusion

Our study showed that collagen type VI is the main component of the subsynovial connective tissue. There is no difference in the expression of collagen type VI in the subsynovial connective tissue in the cases of diabetic CTS and idiopathic CTS. There is no difference between the

two patient groups in terms of the expression of collagen type III expressing in the subsynovial connective tissue in response to injury. Collagen type III is to express more strongly in the vessel wall in the diabetic CTS cases. In the diabetic CTS, TGF- $\beta$  expression in subsynovial connective tissue and vascular walls is more weak according to idiopathic CTS.

In our opinion, the cause of the changes in subsynovial connective tissue is more likely microvascular changes and metabolic factors than TGF- $\beta$  expression in diabetic CTS cases. Thus, TGF- $\beta$  receptor inhibition, which is used to decelerate the progress of the disease or as a therapy option in idiopathic CTS, is going to be useless in diabetic CTS cases.

Subsynovial connective tissue changes and TGF- $\beta$  expression in diabetic CTS awaits more study as the mechanism and the therapy options are different than idiopathic CTS.

Above all, diabetic CTS group must be considered as a different disease groups than the idiopathic CTS group when making studies on CTS and assessing treatment options.

### Summary

In recent, it focuses on the role of histopathological and immunohistochemical changes in the etiopathogenesis of CTS. Studies on collagen remodeling are maintained in the subsynovial connective tissue in CTS. Systemic factors facilitate the formation of the syndrome.

# TGF- $\beta$ expression in the diabetic and idiopathic carpal tunnel syndrome

We evaluated the structure of collagen in the subsynovial connective tissue in diabetic and idiopathic CTS cases. Collagen type VI is seen as a major component of subsynovial connective tissue in the both patient groups. There is no difference between the two patient groups in terms of the expression of collagen type III expressing in the subsynovial connective tissue in response to injury. The expression of collagen type III is more intense in the vascular wall in diabetic CTS. In the diabetic CTS cases, TGF  $\beta$  expression in subsynovial connective tissue and vascular walls seems weakened. The microvascular changes and metabolic factors rather than TGF  $\beta$  expression are considered responsible for changes in the subsynovial connective tissue in diabetic CTS cases.

## Acknowledgements

This research hereby is supported through the Dumlupınar University.

## Disclosure of conflict of interest

None.

## Authors' contribution

Please specify the contribution of each author to the paper, e.g. study design, data collections, data analysis and writing. Ayse Nur Deger: Study design, data analysis, literature search, drafting writing. Hakki Deger: Data collection, Operation of all cases; Mahir Tayfur: Data analysis, literature search; Mecdi Gurhan Balci: Data analysis, literature search; Asude Aksoy: literature search; Mehmet Fatih Ekici: Writing; Melike Nalbant Moray: Literature search.

## Abbreviations

AEC, 3 amino-9 ethyl-carbozole; CTS, Carpal Tunnel Syndrome; DM, Diabetes mellitus; EMG, Electromyography; HRP, Horseradish peroxidase; PBS, Phosphate buffered saline; TGF- $\beta$ , Transforming growth factor- $\beta$ .

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