

Study on the expression level of NF- κ B and clinical evaluations in spinal tuberculosis patients

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ABSTRACT

Spinal tuberculosis or tuberculous spondylitis is one of the most common types of skeletal tuberculosis. Complications of the spine and spinal cord tuberculosis include destruction of the vertebrae, deformity, and paraplegia. Since in some patients, the clinical manifestations of tuberculosis are unusual and timely diagnosis and treatment of this disease can prevent its serious consequences, so in the present study, some cases of rare manifestations of tuberculosis were investigated. The expression of the NF- κ B gene in these patients was also evaluated. In this regard, 36 patients with spinal tuberculosis and 30 healthy individuals (as a control group) were assessed. Clinical symptoms, imaging, laboratory tests, pathology, and response to treatment related to patients with spinal tuberculosis and spinal cord tuberculosis were evaluated. NF- κ B expression was also evaluated using the PCR technique in peripheral white blood cell samples. The obtained results were analyzed using SPSS ver. 16, χ^2 and T-test statistical methods. Mann-Whitney U test and Kruskal-Wallis non-parametric tests were used to analyze non-parametric data. The results showed that out of 36 cases of spinal tuberculosis, 29 cases had spinal tuberculosis, five cases had tuberculous radiculomyelitis, one case had spinal intramedullary tuberculoma, and one case had syringomyelia. 52.78% of patients were male, and 70% of cases were observed between the ages of 35 and 55 years. Fever and back pain were seen in more than 80% of cases. The study of NF- κ B expression in the control and case groups showed that the NF- κ B expression in the case group increased compared to the control group. This increase was statistically significant ($P = 0.0071$). In general, in the present study, the methods of clinical diagnosis of spinal tuberculosis were evaluated. Also, the amount of NF- κ B transcription factor was evaluated as an effective genetic factor in the diagnosis of this disease.

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Introduction

Tuberculosis is one of the most common causes of death in human societies so that in 1993, the World Health Organization (WHO) declared tuberculosis a public health emergency. 10 to 20% of cases of this disease are extrapulmonary tuberculosis (EPTB) (1). But the most complicated form is central nervous system disease, which has severe consequences for the patient if not diagnosed or treated late. Sometimes, due to the ambiguity and unknown clinical symptoms, the diagnosis is made late or even, in some cases, is made incorrect (2). Therefore, clinical awareness about this disease should always be considered to reduce patients suffering from the dangerous consequences (3).

One of the critical types of central nervous system tuberculosis is spinal cord tuberculosis, which is

mainly caused by spinal tuberculosis (tuberculous spondylitis). About one-third of cases of bone tuberculosis appear as spinal tuberculosis. In the early stages of the disease, i.e. the involvement is limited to bone and disc, patients usually complain of pain and tenderness in the affected area (4). As the disease progresses, neurological symptoms of spinal cord injury and its roots appear. But the involvement of the spinal cord during tuberculosis does not end here (5). Its involvement occurs in various forms, such as spinal cord tuberculosis, arachnoiditis, radiculomyelitis, and syringomyelia without vertebral involvement. These complications, which can be a complication of meningitis, are rare and dangerous and can only lead to complete recovery if diagnosed and treated early (2). Tuberculosis can damage enzymes, DNA, and other macromolecules and induce

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oxidative stress responses. The results show that tuberculosis causes changes in immune and inflammatory responses in these patients (5). The NF- κ B factor is known as one of the critical factors in regulating inflammatory and immune responses (6).

The NF- κ B transcription factor was introduced in 1986 as a DNA-binding protein that identifies an essential motif in the electron moiety of the κ -immunoglobulin light chain (7). Subsequent research revealed that this factor is present in different cells, a family of varying homo or heterodimers (Rel family) (8). The transcription factor NF- κ B remains unchanged phylogenetically from insects to mammals, which highlights the importance of this factor. The NF- κ B family consists of five members, including Rel A (p65), Rel B, c-Rel, NF- κ B1 (p50), and NF- κ B2 (p52) (9). These proteins remain inactive in the cell cytoplasm in connection with their inhibitor, i.e., I κ B, to receive the appropriate signal for activation. The NF- κ B is stimulated and activated in response to cellular stimuli. About 450 stimuli, including physical, chemical, physiological, and oxidant, have been identified for NF- κ B (10, 11). Also, mitogens, ligand receptors, bacteria, viruses, parasites, fungi, and their products, proinflammatory cytokines, and some pathological conditions are among the stimuli of this transcription factor (6).

This transcription factor is involved in various cellular activities and plays a vital role in different biological functions. Known actions of this factor include the regulation of immune and inflammatory responses, cell proliferation, and apoptosis (12). NF- κ B activity is essential for hematopoiesis, differentiation, and maturation of both immune cells, namely myeloid and lymphoid cells, including NK cells, B and T cells, dendritic cells, macrophages, and neutrophils. Production of IL-12 by human dendritic cells requires NF- κ B activity (13, 14).

The NF- κ B transcription factor delivers many pro-inflammatory genes, including cytokines, chemokines, immune receptors, enzymes, and other pro-inflammatory molecules. Improper NF- κ B activity is one of the mechanisms of some diseases, especially those associated with inflammation or apoptosis (15, 16). Altered NF- κ B activity can be seen in stroke, Severe epileptic seizures, Traumatic brain injury, Alzheimer's disease, Amyotrophic lateral sclerosis, Parkinson's, Ulcerative gastritis, Crohn's disease,

Huntington's disease, Immune glomerulonephritis, Psoriasis, sunburn, Lyme disease, contact dermatitis, skin cancer, diabetes, etc (12).

Considering the importance of tuberculosis, its high incidence (2), and the role of NF- κ B transcription factor in the expression of cytokines (15), we decided to conduct a study in patients with tuberculosis of the spine and spinal cord. The study aimed to assess clinical manifestations and evaluate the expression of NF- κ B transcription factors to prevent severe complications by early diagnosis of the disease.

Materials and methods

Demographic and clinical evaluations

This case-control study was performed on 36 patients with spinal tuberculosis and central nervous system tuberculosis (radiculomyelitis, spinal tuberculosis, and syringomyelia). The control group also included 30 healthy individuals with the same age, gender, weight, and race as the patients and without family relationships with the patient group members. Demographic data, including age, gender, and the main complaint when patients were referred, were recorded in a questionnaire. Clinical examination assessments included the presence or absence of kyphosis, musculoskeletal abnormalities, sphincter abnormalities, and Babinski reflex symptoms in all patients. Clinical, laboratory, pathological, and imaging findings were recorded. For two months, all patients were treated with the four drugs isoniazid, rifampin, ethambutol, and pyrazinamide.

RNA extraction

After obtaining written consent, 5ml of peripheral blood was collected from the subjects in EDTA Vacutainer tubes (Bio-Sciences, USA). The RNA was extracted using an RNA extraction kit (Thermo Fisher Scientific, USA) according to the company's instructions, which included the following steps:

1. Homogenize the sample using RNA solution
2. RNA extraction using chloroform
3. RNA deposition using isopropanol
4. RNA elution using 75% ethanol

After RNA extraction, the prepared samples were immediately placed in liquid nitrogen to be transferred to the laboratory.

RT-PCR for cDNA synthesis

By the RT-PCR method, RNA samples were converted to cDNA. For this purpose, 2 μ l of extracted RNA and 1 μ l of Oligo-dT primer were added to a sterile tube. The tube was placed at 70°C for 5 minutes and immediately transferred to ice. The resulting mixture was transferred to a micro-tube containing AccPower RT PreMix (Bioneer, USA) and diluted to 50 μ l with DEPCIII double distilled water. The lyophilized sediment at the bottom of the micro-tube was dissolved by gentle blows and thoroughly mixed with the solution, and the cDNA synthesis reaction was performed as follows: 60 minutes at 42°C for cDNA synthesis, and 5 minutes at 94°C to disable Reverse Transcriptase.

Primer design and PCR

By the data recorded in Genbank, complete sequences of NF- κ B and β -actin (housekeeping gene as internal control) genes were extracted, then by Primer3 software, the desired primer pairs were designed to amplify these two genes. Sequences used to synthesize the genes are mentioned in Table 1. PCR reaction was used to study the expression of NF- κ B and β -actin genes. The program used is given in Table 2.

Table 1. Primer sequences and annealing temperature for NF- κ B and β -actin genes; forward (A) and reverse (B), annealing temperature (#)

Gene		Primer Sequence	#
NF- κ B	A	5'-CAAGGCAGCAAATAGACGAG-3'	46°C
	B	5'-GTTGAGAGTTAGCAGTGAGGCA-3'	
β -actin	A	5'-CCTGGAGGAGAGCTACGAG-3'	49°C
	B	5'-TTCATGATGGAGTTGAAGGT-3'	

Table 2. The Polymerase Chain reaction (PCR) program for NF- κ B and β -actin genes

Stage	Temperature (°C)	Time	Number of Cycle
Initial denaturation	94	2 minutes	1
Denaturation	94	30 seconds	30
Annealing	46 - 49	40 seconds	30
Extension	72	40 seconds	30
Final Extension	72	5 minutes	1

Electrophoresis and analysis of PCR products

The product obtained from PCR reaction was electrophoresed on 2% gel and stained with ethidium bromide. The prepared gels were examined and photographed by Gel Documentation. The prepared photos were saved for further evaluations. In the next

step, the images were analyzed by Totallab Quant software (v: 11.5).

Statistical analysis

The obtained results were analyzed by SPSS software version 19, and statistical methods such as the χ^2 test and T-test. Mann-Whitney U test and Kruskal–Wallis non-parametric test were also used to analyze non-parametric data.

Results and discussion

Demographic and clinical results

Out of 66 people who participated in this study, 30 were in the control group, and 36 were in the case group. In the control group, the mean age was 50.6 years with a standard deviation of 10.5 years, and in the case group, the mean age was 54 years with a standard deviation of 9.1 years. The two groups did not differ significantly in terms of age ($P = 0.08$). Also, 56.67% of the control group and 52.78% of the case group were males, which in this regard, there was no significant difference between the two groups ($P = 0.13$).

Among the 36 patients with spinal tuberculosis, 70% of patients ranged from 35 to 55 years. Fever above 37.8°C was the most common clinical sign (89%). Back pain was seen in 83% of cases. Other clinical manifestations are shown in Table 3.

Table 3. Frequency of clinical symptoms in 36 patients with spinal tuberculosis

Clinical Symptoms	Absolute Frequency	Relative Frequency (%)
Fever	32	89
backache	30	83
Limb pain	16	44
Kyphosis	16	44
Sweating	15	42
Anorexia	15	42
Cough	14	39
Limb weakness	14	39
Paralysis of the limbs	9	25
Babinski reflex symptoms	7	19
urinary incontinence	5	14
Stool incontinence	3	8

Fifteen patients (42%) had tuberculosis of other organs (Multi organ), of which nine cases had pulmonary tuberculosis and six cases had tuberculous

lymphadenitis. In the 36 patients studied, 64% had normal white blood cell count (WBC), 28% had increased white blood cell count (leukocytosis), and 8% had decreased white blood cell count (leukopenia). In 87% of patients, the rate of globular deposition was above 40mm in the first hour (mean = 58mm). The tuberculosis skin test (also known as the tuberculin or PPD test) was greater than 10 mm in 81% of cases. Eight (22%) of the 36 patients were required surgery. Twenty-nine patients (80%) (Including two patients with spinal tuberculosis and 27 patients with tuberculous spondylitis) responded appropriately to medical treatment alone or with surgery, and the disease improved without complications. 20% of cases had improvement with complications.

These 36 patients were divided into two groups. One group consisted of 29 cases of tuberculous spondylitis, and the other group consisted of seven cases with spinal cord tuberculosis without vertebral involvement.

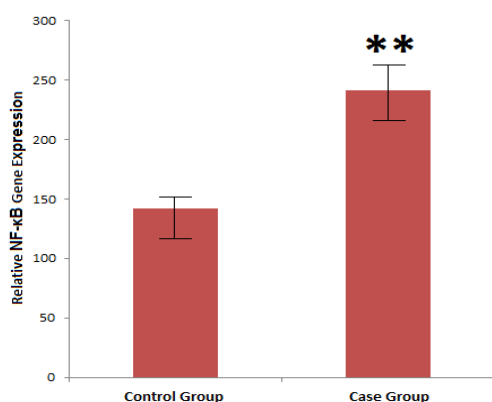


Figure1. Relative expression of NF-κB gene in control and case groups

A. 29 patients with spinal tuberculosis

According to the radio graphics and CT scans results, among 29 patients with spinal tuberculosis, the most vertebral involvement was related to simultaneous involvement of the lumbar vertebrae (12 cases), dorsal vertebrae alone (eight cases), Lumbar vertebrae alone (five cases), cervical vertebrae alone (two cases), dorsal cervical vertebrae (one case), and sacral vertebrae (one case). Of the 29 patients with spinal tuberculosis, 13 cases were taken from the involved vertebrae. In nine cases, the pathological symptoms were consistent with the diagnosis of tuberculosis (granulomas with or without caseous). Smear and tissue aspiration cultures were positive for

tuberculosis in four cases of patients with spinal tuberculosis.

B. Seven patients with spinal cord tuberculosis

Out of seven cases of spinal cord tuberculosis, five patients had tuberculous radiculomyelitis, one case had intramedullary colic tuberculosis, and one case had spinal syringomyelia with tuberculous meningitis. There were five cases of tuberculous radiculomyelitis (three females and two males) without vertebral involvement, which three cases had tuberculous meningitis. Clinical signs of the disease included fever, low back pain, paraplegia, sphincter disorder, and positive Babinski reflex symptoms. Definitive diagnosis was given in three cases by positive PCR (Polymerase Chain Reaction) test in cerebrospinal fluid. In one case, positive culture of tuberculosis bacteria in cerebrospinal fluid was reported. The diagnosis was confirmed in the latter case due to the association of pulmonary tuberculosis, positive sputum smear, and response to treatment (17). The sixth person in this group was a rare case of intramedullary cervical tuberculoma. She was a 21-year-old woman who was initially hospitalized with a diagnosis of tuberculous meningitis. After initiating treatment and establishing a ventricular-peritoneal shunt, the patient developed low back pain, paralysis of lower limbs, urinary incontinence, and Babinski reflex symptoms. By myelography, the clinical suspicion of tuberculosis was raised, and after surgery and removal of the spinal cord compression effect, tissue was sampled. In pathology, caseous granuloma was reported. The seventh case was a rare case of spinal syringomyelia following tuberculous meningitis. A 27-year-old woman with symptoms of headache, fever, neck stiffness, paralysis, and diplopia was referred. This patient was confirmed for TB by positive spinal fluid PCR test, and the intrathecal injury was observed in the lower part of the dorsal-lumbar spinal cord in myelography. At the end of the surgery, Syrix was diagnosed within a spinal cord.

Genetic analysis results

NF-κB expression in the control and case groups showed that the mean of NF-κB in the case group was 241.75 and in the control group was 141.99, which indicated increases in the NF-κB expression of the

case group. This increase was statistically significant ($P = 0.0071$)(Figure 1).

Tuberculosis is one of the ten leading causes of death worldwide. The disease is caused by different species of Mycobacteria, commonly known as *Mycobacterium tuberculosis* (17). There are different types of this disease, which spinal tuberculosis is one of them. It affects young and middle-aged people in poor communities but is more common among the elderly in rich communities (18). In the present study, spinal tuberculosis was reported in the age range of 35 to 55 years. The clinical manifestations of this type of tuberculosis are Low back pain, Muscle stiffness around the spine, and Pain sensation on clinical examination. In this study, fever, back pain, and limb pain were the most common clinical symptoms in the patients. Over time, bone destruction and destruction of the intervertebral disc occur.

In this study, plain radiography was helpful only in patients with spinal tuberculosis. The first site of involvement is usually the upper anterior and lower anterior angles of the vertebral body, which propagate to the intervertebral disc and adjacent vertebrae. This disease can lead to disc and vertebral destruction, neurological complications, and humpback deformity. The dorsal and lumbar spines are more involved than other vertebrae, followed by the cervical and sacral vertebrae. Simultaneous involvement of the lumbar spine in 41% (most common) and involvement of the dorsal vertebra in 27.5% of cases were seen in the patients of this study.

In a study on 69 cases of spinal tuberculosis over 14 years, the mean age of patients was 52.8 years, and 53.6% were male (19). Fever and vertebral sensitivity were the most common symptoms in these patients. Paraparesis and kyphosis were other symptoms of the disease. In 94.5% of cases, the deposition rate in the first hour was higher than 20 mm, and in 73.2% of cases, more than 40 mm. This finding is consistent with current research that the deposition rate in 80% of patients was higher than 40 mm/hour. CT scan and MRI reported thoracic spine involvement in 55% of cases. In the current study, 27.5% of patients showed spinal cord involvement. In 45% of cases, the dorsal vertebrae were involved with other vertebrae (12 in the back and 1 in the neck). In another 27.5% of cases, there was the involvement of other vertebrae.

In a study by Konbaz et al. (19), 54% of patients responded to medical treatment, and 46% needed surgery. Complete recovery was seen in 91% of cases. In the present study, among 29 patients with spinal tuberculosis, improvement was seen in 27 patients (93%) because the diagnosis was based on clinical findings and radiography, while in seven patients with spinal tuberculosis, Due to the involvement of vertebrae in plain radiography, late diagnosis of disease and spinal cord injury, recovery was seen in only two cases out of seven patients (28.5%). Another study by Turgut et al. (20), which was performed on 694 patients with spinal tuberculosis, had the initial symptoms of weakness and paresis of the legs (69%), humpback deformity (46%), and low back pain (21%). The symptoms of kyphosis, weakness, and paralysis of the limbs were seen in 44%, 39%, and 25% of patients, respectively. This study identifies spinal tuberculosis as one of the most important causes of paraplegia in Turkey. It suggests that this disease should be considered in any patient with neurological findings due to spinal cord pressure and spinal deformity. The prognosis of spinal tuberculosis is good if the compressive effect on the spinal cord is quickly eliminated (21). A similar study in Senegal found that Pott's disease, or spinal tuberculosis, was the number one cause of spinal strain (22). In another study conducted by Yadav et al. (23) in adults, 50% of cases had tuberculosis with multiple organ involvement, and in 61.2% of cases, two adjacent vertebral bodies were involved. Neurological defects were seen in 68% of cases.

Based on the Frankel system, Vidyasagar and Murthy (24) divided the neurological symptoms of vertebral tuberculosis into five groups and reported a study of 200 cases of vertebrate tuberculosis patients with neurological complications. They considered myelography to be the best method of diagnosis when there is pressure on the spinal cord and prescribed anti-tuberculosis and surgical drugs to treat these cases (24). In another study, MRI reported a better way to diagnose (25). In the current study, myelography was performed in seven cases of spinal cord tuberculosis and two cases of spinal tuberculosis, which helped diagnose the disease.

Eisen et al. (26) showed that the prognosis for spinal tuberculosis was better in children than in adults. Patients with chronic paralysis for six months

did not show neurological recovery after surgery. Therefore, the decision to have surgery should be made as soon as possible (27). Because in this disease, the process of tuberculosis infection and the compressive effect on the spinal cord coincide, so simultaneous medical treatment and surgery are recommended. In 29 patients, Machida suggested the addition of steroids in such cases (28). There are numerous reports of rare forms of spinal tuberculosis. Less common forms of spinal cord involvement are intramedullary tuberculosis, intradural tuberculoma, syringomyelia due to tuberculous meningitis, and tuberculous radiculomyelitis (29).

These cases usually require surgery in addition to anti-tuberculosis medication but are less likely to respond to anti-tuberculosis medication and corticosteroids alone (30). A case of tuberculosis has been reported in a 46-year-old man who presented with weakness and paralysis of the left leg. He noted the history of tuberculosis from 10 years ago (30).

Varghese et al. (31) also reported a case of intramedullary tuberculosis in a 20-year-old man who presented with progressive leg weakness, impotence, and urinary problems similar to Conus Tumor, and was confirmed by MRI and pathology, tuberculosis. Tan et al. (32) reported a case of spinal intramedullary Coloma tubercle with syringomyelia in the thoracic spinal cord, which was diagnosed by MRI and responded well to anti-tuberculosis drug therapy. While in the patient of the present study, spinal tuberculosis was associated with arachnoiditis and tuberculous meningitis. Despite the treatment of tuberculous meningitis, spinal cord and conus involvement symptoms appeared which was confirmed in the pathology report of tuberculoma.

Regarding genetic evaluations, the expression of the NF-κB transcription factor was investigated in the present study. Peripheral blood cells of patients with spinal tuberculosis were evaluated to evaluate NF-κB gene expression. The results showed that the amount of this factor in these cells increased compared to the control group, and this increase was statistically significant. NF-κB is a transcription factor that plays an essential role in inflammation and immune responses. The NF-κB factor is involved in different stages of differentiation of B cells and T cells. This factor also plays a role in many diseases and its expression changes. This factor increases in some of

these diseases, including cystic fibrosis, asthma, chronic heart failure, diabetes, kidney disease, lung disease, and cancer (33-36).

In general, in the present study, the methods of clinical diagnosis of spinal tuberculosis were evaluated. Also, the amount of NF-κB transcription factor was evaluated as an effective genetic factor in the diagnosis of this disease.

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