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Original Article

Role of nerve growth factor in allergic and inflammatory lung diseases

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Background and Purpose: Nerve growth factor (NGF) is a neurotrophin that plays an important role in the development and function of the central and peripheral nervous systems. We investigated the role of NGF receptors in allergic and inflammatory lung diseases.

Methods: This study included 90 children who attended the outpatient pediatric clinic or who were admitted to the inpatient pediatric department of El-Minia University Hospital. The children were divided into 3 groups — Group I, asthmatic children who had sustained an acute attack; Group II, children with severe inflammatory lung disease such as bronchopneumonia; and Group III, 20 apparently healthy children who were age- and sex-matched to the diseased groups. Thorough clinical examination, chest X-ray, complete blood count, erythrocyte sedimentation rate, and reverse transcriptase-polymerase chain reaction (RT-PCR) were carried out.

Results: RT-PCR revealed only 3 asthmatic cases that showed positive NGF receptors on isolated eosinophils from the peripheral blood. However, all cases with bronchopneumonia had no detectable results. Moreover, there was a statistically significant difference between positive and negative cases for NGF receptors on isolated eosinophils by RT-PCR with regard to age ($p<0.001$), frequency of recurrence of asthmatic attacks ($p<0.005$), positive history of other atopic diseases such as allergic dermatitis, and allergic rhinitis ($p<0.02$). However, there was no statistically significant difference between positive and negative cases with respect to sex, type of feeding, and/or family history.

Conclusions: There is a strong association between NGF receptors on isolated eosinophils and the severity of allergic lung diseases and bronchial asthma.

Key words: Eosinophils, lung diseases, nerve growth factor, reverse transcriptase polymerase chain reaction

Introduction

Nerve growth factor (NGF), a neurotrophin, is known to induce growth and differentiation of neurons. It plays an important role in the development and function of the central and peripheral nervous systems. However, it was recently reported that several immune cells such as mast cells, lymphocytes, and eosinophils produce, store, and release NGF [1]. Inflammatory lung diseases represent a group of severe diseases with increasing prevalence as well as epidemiological importance. Inflammatory lung diseases could result from allergic or infectious genesis. There is growing evidence that immune and nervous systems are closely related not only in physiological but also in pathological reactions in the lung [2]. Extensive communications between neurons and immune cells are responsible for the magnitude of airway inflammation and the development of airway hyperreactivity, a consequence of neuronal dysregulation [3]. Neurotrophins also simultaneously exhibit profound effects on immune cells residing in the airways and lung tissue, thus acting as amplifiers of the locally occurring immune imbalance. This effect has so far been exclusively demonstrated for NGF. NGF augments the production of interleukin-4 and interleukin-5, but not interferon on activation of lymphocytes by allergens. Furthermore, these increases result in enhanced immunoglobulin E antibody levels [4].

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Neurotrophin receptors are expressed on central and peripheral neurons, lymphocytes, monocytes, mast cells, and fibroblasts [5]. Neurotrophins bind to 2 classes of receptors — tyrosine kinases encoded by the trk gene family and P75 glycoprotein (p75NTR) [5]. The biological effects of NGF are mediated by 2 classes of receptors. The p75NTR belonging to the superfamily of tumor necrosis factor receptors, to which all members of the neurotrophin family bind with similar affinity, has also been suggested to function as a co-receptor for the transmembrane tyrosine kinase (trkA) receptor tyrosine kinases [4]. Recent evidence from animal models of allergic asthma indicates that p75NTR plays a critical role in the accumulation of eosinophils in the lung. Also, blocking of p75NTR by local antibody treatment prevents eosinophilic lung inflammation in a murine asthma model [6] and a trkA of 140 kDa [7]. It has been identified as the preferred receptor for NGF, and its stimulation is necessary and sufficient to elicit a full biological response to NGF in different cell types [8].

Methods

This study included 90 children who attended the outpatient pediatric clinic or who were admitted to the inpatient pediatric department of El-Minia University Hospital between April and November 2004. They were classified into 3 groups. Group I included 35 cases of asthmatic children (22 males, 63%; 13 females, 37%) who had sustained an acute attack. Their ages ranged from 1 to 8 years (mean ± SD, 4.6 ± 1.9 years). Group II included 35 cases (20 males, 57%; 15 females, 43%) who required medical attention for bronchopneumonia, a severe inflammatory lung disease. Their ages ranged from 1 to 12 years (mean ± SD, 4.5 ± 1.8 years). Group III included 20 apparently healthy children who were age- and sex-matched to the diseased groups. Their ages ranged from 1 to 8 years (mean ± SD, 3.9 ± 1.4 years).

For all patients and controls, the following parameters were recorded: name, age, sex, type of feeding, number of wheezing attacks, family history of asthma, and/or other allergic disorders. Data also included information on history of fever, chills, cough, unusually rapid breathing with or without grunting, cyanosis, poor feeding, and other symptoms suggestive of bronchopneumonia. Thorough clinical examination, chest X-ray (posteroanterior and lateral views), and laboratory investigations including a complete blood count and erythrocyte sedimentation rate (ESR) at 1 and 2 h (Westergren method) were also carried out.

Extraction of RNA from isolated eosinophils

Venous blood samples (4 mL) were collected from all patients and controls by venipuncture under aseptic conditions into a polypropylene tube containing ethylenediamine tetra-acetic acid (EDTA).

Eosinophils were purified from peripheral blood samples by negative selection using anti-CD16-bound immunomagnetic beads and a magnetic cell sorting system. The preparations contained more than 98% eosinophils and the contaminating cells were neutrophils.

Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) was applied to detect NGF receptor mRNA expression in purified eosinophils obtained from peripheral blood.

This was attempted using spin columns from the QIAamp DNA Mini Kit and QIAamp DNA blood Mini Kit. RT-PCR was carried out using Reverse-iT™ the 1st strand synthesis kit (ABgene, Epson, UK) by the one-step method. The transcript DNA was further used for PCR reaction, using the sense (CCTACGGCTACTA CCAGGATG) and antisense (TGGCCTCGTCGGA ATACG) primers of p75NTR. The PCR reaction was established using PCR mixture containing 34.5 μL of sterile water, 5 μL of 10x amplification buffer, 4 μL of a mixture of dNTPS, and 2 μL of magnesium chloride in a total volume of 45.5 μL. DNA (1 μL) was added to the latter PCR mixture, followed by the addition of 2.5 μL of the primer in the total volume of 50 μL. Finally, 200 μL of ultra-pure mineral oil was added to the mixture, which was then applied to the PCR machine. After an initial denaturation step at 95°C for 10 min, the PCR reaction was performed at an annealing temperature of 60°C for 10 sec, followed by an extension phase at 72°C for 9 sec, and a denaturation cycle for 1 sec. After cooling to 40°C, 5 μL of each reaction was run on a 2% agarose gel and visualized with ethidium bromide.

Statistical analysis

Statistical analysis was done by unpaired *t*-test between 2 groups and paired *t*-test within the same group. For non-parametric data, Mann-Whitney *U* test was performed between groups (equivalent to *t*-test). Chi-squared tests were used to compare non-parametric data (student's *t* and chi-squared tests). Spearman's rank correlation was used to measure the relationship between variables or rank orders.

Table 1. Demographic data of patients with bronchial asthma, bronchopneumonia, and controls

Parameter	Group I: Asthmatic cases (n = 35) No. (%)	Group II: Cases with bronchopneumonia (n = 35) No. (%)	Group III: Control group (n = 20) No. (%)	*p*		
				Group I vs III	Group II vs III	Group I vs II
Age (years)						
Range	1-8	1-12	1-8	0.111	0.224	0.814
Mean ± SD	4.6 ± 1.9	4.5 ± 1.8	3.9 ± 1.4			
Gender						
Male	22 (63)	20 (57)	12 (60)	0.84	0.84	0.63
Female	13 (37)	15 (43)	8 (40)			
Breast feeding	20 (57.1)	25 (71.4)	15 (75)	0.189	0.77	0.27
Artificial feeding	15 (42.9)	10 (28.6)	5 (25)			
Family history of similar conditions						
Positive	18 (51.4)	0 (0)	0 (0)	0.000[a]	1	0.000[a]
Negative	17 (48.6)	35 (100)	20 (100)			
Family history of other atopy conditions						
Positive	21 (60)	0 (0)	0 (0)	0.001[a]	0.54	0.007[a]
Negative	14 (40)	35 (100)	20 (100)			
Recurrences of similar conditions						
Range	0-7	0-3	0	0.000[b]	0.01[a]	0.000[a]
Mean ± SD	1.7 ± 1.8	0.8 ± 0.4	0			

Abbreviation: SD = standard deviation

[a]Significant.

[b]Highly significant.

Results

Table 1 lists the history of atopic disorders and the number of recurrences of similar conditions in the different groups studied. There was no statistically significant difference in age between the different study groups. A statistically significant difference was found in the presence of positive family history of bronchial asthma ($p<0.001$) among groups I, II, and III. Moreover, a statistically significant difference ($p<0.01$) was found with regard to the presence of other atopic disorders between groups I, II, and III. There was a statistically significant difference in the recurrence of similar conditions between group I and the control group ($p<0.000$). Similarly, it was observed that there was a statistically significant difference between groups II and III ($p<0.001$). However, there was no statistically significant difference between the 3 study groups with respect to sex or type of feeding. The frequency of both positive and negative cases for NGF receptors on isolated eosinophils by RT-PCR for the different groups is shown in Table 2.

Table 3 illustrates the relationship between age and the positive and negative cases for NGF receptors on isolated eosinophils by RT-PCR in group I ($p<0.001$). Table 3 also shows the relationship between the presence of other atopic disorders in asthmatic children (group I) and positive and negative cases for NGF receptors on isolated eosinophils by RT-PCR. The resulting data (Table 3) shows that there was a statistically significant increase ($p<0.001$) between age and the frequency of the recurrence of asthmatic attack. In addition, Table 3 also shows that there was a statistically significant increase in the frequency of the recurrence of asthmatic attack in positive cases for NGF receptors by RT-PCR as compared to that in the negative cases ($p<0.005$).

Table 2. Frequency of positive and negative cases for nerve growth factor receptors on isolated eosinophils in different groups of the study

RT-PCR	Group I (n = 35) No. (%)	Group II (n = 35) No. (%)	Group III (n = 20) No. (%)
Negative cases	32 (91)	35 (100)	20 (100)
Positive cases	3 (9)	0 (0)	0 (0)

Abbreviation: RT-PCR = reverse transcriptase-polymerase chain reaction

Table 3. Comparison between positive and negative cases for nerve growth factor receptors on isolated eosinophils in group I with respect to age, number of recurrences, and presence of other atopies

	Positive cases (n = 3)	Negative cases (n = 32)	*p*
Age (years)			
Range	3-5.2	1-4	0.001[a]
Mean ± SD	4.2 ± 0.9	2.2 ± 0.9	
No. of recurrences			
Range	4-7	0-4	0.005[a]
Mean ± SD	5.3 ± 1.5	1.5 ± 1.4	
Other atopy			
Present (%)	3 (100)	11 (34.4)	0.02[a]
Absent (%)	0	21 (65.6)	

Abbreviation: SD = standard deviation
[a]Significant.

Table 3 also shows the relationship between the presence of other atopic disorders in positive, group I, and negative cases for NGF receptors on isolated eosinophils by RT-PCR.

Table 4 shows the correlation between positive cases in group I for NGF receptors by RT-PCR and age, number of asthmatic attacks, and history of other atopic disorders. It is indicated that there is a significant positive correlation between the ages, frequency of asthmatic attacks, history of other atopic disorders, and positive cases for NGF receptors on the isolated eosinophils [Table 4]. Table 5 shows the results obtained by comparison between the positive and negative cases for some hematological findings and between positive cases and group I for NGF receptors. It is shown that there is no statistically significant difference between the 2 groups with regard to the hematological findings studied, except the isolated eosinophil count that was high in positive cases ($p<0.005$). Table 5 also reveals the comparison between positive and negative cases for NGF receptors in group I with regard to ESR at 1 and 2 h. A non-significant difference was found in ESR between positive and negative cases. The results of eosinophil count (cells/mL) against RT-PCR results (positive and negative cases) are shown in Fig. 1 and Table 5. mRNA expression of neurotrophin receptors in peripheral blood (P75 NTP) is shown in Fig. 2. The isolated band is 147 bp, which is present in lanes 11, 22, and 24 with 100 bp DNA ladder. The frequency of atopy showed a significantly higher number of positive cases of NGF-isolated receptors recorded as a positive history of other atopic disorders (Fig. 3). It was also noted that there was a higher number of atopic manifestations in the 3 positive RT-PCR patients as compared with the negative RT-PCR patients. Fig. 4 shows the diffference between breast-fed children and those who were on artificial feeding. According to the RT-PCR results, the number of positive cases in breast-fed children was less than those on artificial feeding.

Table 4. Correlation between positive cases in group I for nerve growth factor receptors on isolated eosinophils by reverse transcriptase-polymerase chain reaction and age, number of asthmatic attacks, and history of other atopic disorders

	r-value	*p*
Age	0.446	0.007[a]
No. of asthmatic attacks	0.478	0.004[a]
History of atopic disorders	0.375	0.02[a]

[a]Significant.

Discussion

Neurotrophins have been observed in elevated concentrations in several inflammatory conditions [9] and have recently been described in allergic diseases [10]. They are a group of structurally related proteins that includes NGF, brain-derived neurotrophic factor, and neurotrophin 3, 4, and 5 [8]. NGF is the best characterized member of the neurotrophin family [11]. NGF receptors are expressed on central and peripheral neurons, eosinophils, lymphocytes, monocytes, mast cells, and fibroblasts. Based on the distribution of its receptors, NGF controls the development and function of neurons and regulates inflammatory processes [5]. Mast cells and eosinophils are key cells of allergic inflammation that secrete NGF [11].

The role of NGF in allergic inflammatory diseases can be explained as follows: NGF induces neuronal changes (increased excitability, hypersensitivity, and induction of neuropeptide production) during an allergic immune response [12]. Through these neuronal modifications, NGF can induce airway hyperresponsiveness to the electrical field of simulation [12]. Moreover, the proinflammatory properties of NGF include the development, differentiation, chemotaxis, mediator release from inflammatory cells, facilitation of mast cell degranulation, activation, survival of eosinophils and neutrophils, and induction of B-cell differentiation into immunoglobulin-secreting plasma cells as well as fibroblast activation through a complex network influenced by other proinflammatory cytokines [1].

We analyzed NGF receptor expression on peripheral blood eosinophils from patients with allergic asthma and

Table 5. Comparison between some hematological findings and both positive and negative cases in group I for nerve growth factor receptors on isolated eosinophils by reverse transcriptase-polymerase chain reaction

Variable	Positive cases	Negative cases	*p*
Hb (g/dL)			0.537
Range	9.8-13	6.8-18.9	
Mean ± SD	10.9 ± 1.8	11.8 ± 2.2	
Platelet count (x10^3/mL)			0.972
Range	410-473	167-797	
Mean ± SD	433 ± 34.8	429.6 ± 167.9	
Total leukocyte count (x10^3/mL)	6.08-12.7	3.9-19.3	0.591
Range	9.3 ± 3.3	10.7 ± 4.4	
Mean ± SD			
Neutrophils (cells/mL)			0.213
Range	23-41	14-91	
Mean ± SD	34.3 ± 9.9	46.8 ± 16.5	
Eosinophils (cells/mL)			0.005[a]
Range	9-21	0-14	
Mean ± SD	13.3 ± 6.7	2.8 ± 3.1	
ESR at 1 h			0.951
Range	22-30	3-100	
Mean ± SD	25.7 ± 4	25 ± 17.3	
ESR at 2 h			0.779
Range	35-65	6-115	
Mean ± SD	49.3 ± 15	45.3 ± 24.5	

Abbreviations: Hb = hemoglobin; SD = standard deviation; ESR = erythrocyte sedimentation rate
[a]Significant.

those having bronchopneumonia, a severe inflammatory lung disease. RT-PCR revealed only 3 asthmatic cases that had positive NGF receptors on isolated eosinophils from peripheral blood (Fig. 2 and Table 2). This is in agreement with the data reported by Noga et al [13], and indicated that the NGF receptor expression is on peripheral blood esoinophils from patients with mild eosinophilia and a history of allergic disease.

It was reported that neurotrophins had no measurable effects on peripheral blood eosinophils,

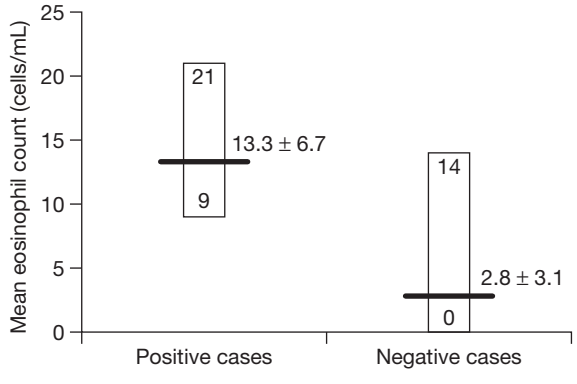

Fig. 1. Eosinophil count according to reverse transcriptase-polymerase chain reaction (RT-PCR) results. Graphics show mean ± standard deviation.

but positive NGF receptors were found on eosinophils from bronchoalveolar lavage fluid [14]. However, we found that all the cases studied with bronchopneumonia gave negative results for NGF receptors on isolated eosinophils from peripheral blood by RT-PCR. These results are in agreement with those reported by Micera et al [11], who studied the involvement of NGF in cases of only allergic inflammation. However, these results differ from those reported by Hoyle [5] suggesting that NGF acts as a mediator of bronchopneumonia.

There was a statistically significant positive correlation between age and positive detection of NGF receptors on isolated eosinophils in group I (p=0.001) [Table 3]; these results contradict those obtained in studies by Nassenstein et al [14] and Kerzel et al [6], in which the age of the groups studied was not considered as a parameter of comparison. Our results also contrast with the findings obtained by Hu et al [15], that the expression of NGF and its receptors decreases progressively with age, but respiratory syncytial virus infection interferes with this physiological decline, promoting a large increase in the expression of both NGF and neurotrophin receptors [15].

There were fewer breast-fed children who had positive results for NGF receptors on isolated eosinophils as

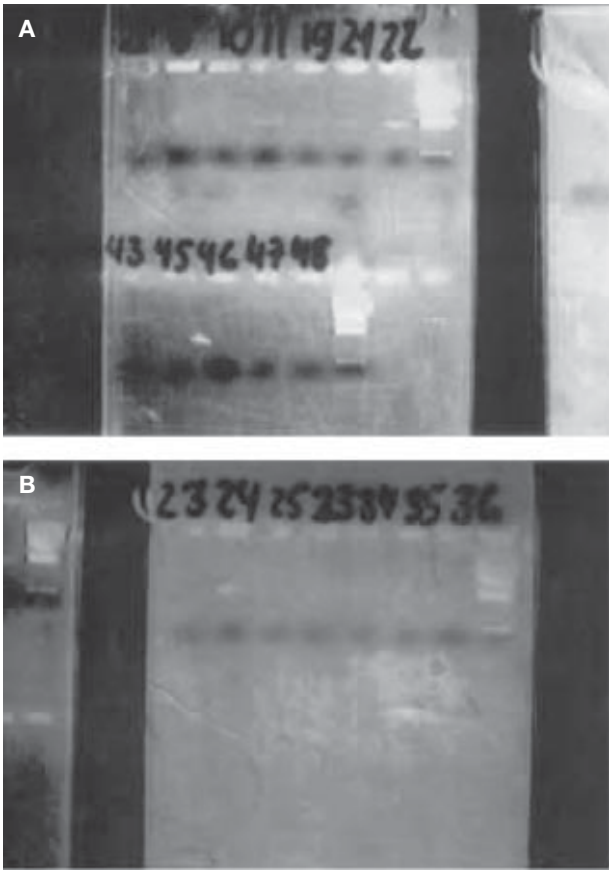

Fig. 2. Positive reverse transcriptase-polymerase chain reaction results in asthmatic cases. The isolated band is 147 bp, which is present in lanes 11, 22 and 24 with 100 bp DNA ladder.

compared to those who were artificially fed (Fig. 4); this was in agreement with the results obtained by Newburg [16], who reported that breast milk contains substances acting as anti-allergens, contributing to a lower incidence of various forms of allergy in breast-fed babies. Also, exclusive breast feeding during the first months after birth is associated with lower asthma rates during childhood. With respect to other atopic disorders, it was found that there was a statistically significant increase in the number of cases in group I as compared to group II and group III ($p<0.001$ and $p<0.01$, respectively; Table 4). The results obtained are in agreement with those reported by Sly [17], which indicated that the term "atopy" implies a hereditary factor expressed as susceptibility to hay fever, asthma, and eczematoid dermatitis in affected individuals.

Furthermore, there was a significantly higher number of positive cases of NGF receptors on isolated eosinophils in patients who reported a positive history of other atopic disorders ($p<0.05$), as indicated in Fig. 3. This is in agreement with the results shown by Sanico

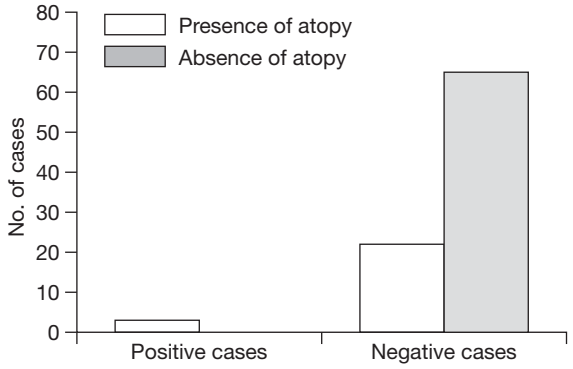

Fig. 3. Reverse transcriptase-polymerase chain reaction (RT-PCR) results in asthmatic cases.

et al [18], Kobayashi et al [19], and Nassenstein et al [14], which indicate that human eosinophils secrete NGF in response to various immunologic stimuli such as allergic asthma, allergic rhinitis, and atopic dermatitis.

However, there was no statistically significant difference between positive and negative cases for NGF receptors on isolated eosinophils with regard to hemoglobin level. Moreover, a significantly higher total leukocyte count was found in groups I and II than in group III ($p<0.01$), and this could be explained by the fact that most bacterial infections of the respiratory tract are accompanied by leukocytosis [20]. Also, the higher total leukocyte count in group I may be explained by the associated upper respiratory tract infections in most asthmatic cases [21]. In spite of the significant difference between the patient and control groups with regard to the total leukocyte count, there was no statistically significant difference between positive and negative NGF receptor status on isolated eosinophils with respect to the total leukocyte count. Also, a higher eosinophil count was found in group I than in group III

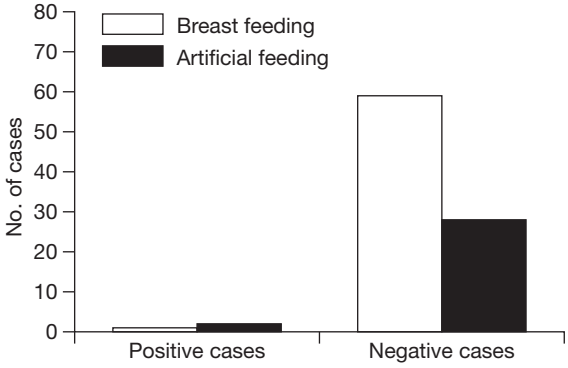

Fig. 4. Reverse transcriptase-polymerase chain reaction (RT-PCR) results for type of feeding.

and the control group, although it was not statistically significant ($p<0.5$). This is in agreement with the results reported, which indicated that eosinophils are subject to diurnal rhythm, their count being higher in the early morning; thus, eosinophilia may be intermittent. Two or three normal results should be obtained before concluding the absence of eosinophilia. However, there was a significantly higher number of eosinophils in cases positive than in those negative for NGF receptors ($p<0.01$) [Table 5]. The former was in agreement with the results obtained by Nassenstein et al [14]. Moreover, Kobayashi et al [19] reported that eosinophils stimulated with immune complexes have an enhanced ability to produce and secrete NGF. Another original finding is that human eosinophils tend to store synthesized cytokines [22]. Moreover, it was reported that NGF might be one such cytokine [19].

In our study, the values of ESR were significantly higher at 1 h and 2 h in the diseased groups, (groups I and II) than in the control group ($p<0.001$), and this is in agreement with Rebbecca [23] and Keith [24], who stated that a mild increase in ESR is correlated with bacterial infections and infected asthma. However, in our study there was no statistically significant difference between positive and negative cases for NGF receptors on isolated eosinophils with respect to ESR ($p<0.77$) [Table 5]. These results were in agreement with those indicating non-significant differences between positive and negative cases with respect to ESR, and this can be explained by the absence of infection [14]. There was no statistically significant difference between positive and negative cases with respect to radiological findings on chest X-ray, and this was in agreement with Nassenstein et al [14]. In conclusion, there is a strong association between NGF receptors on isolated eosinophils and the severity of allergic lung diseases and bronchial asthma.

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