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Original article

Interleukin 23 and Interleukin 17 Evaluation Before and After Methotrexate Treatment of Basal Cell Carcinoma

Nelly Mohamed Said [*1], Shrook Abd ELshafy Khashaba [2], Ahmad Mohamed Elgarf [2], Dina Esmat Hamed [2], Kamal Ahmed Elkashishy [1],

1 Departement of Pathology, Faculty of Medicine, Zagazig University Egypt . 2Departement of Dermatology, Venereology and Andrology, Faculty of Medicine, Zagazig University,Egypt

Corresponding author*

Nelly Mohamed Said M.

Email:

nellyms84@gmail.com

Pathology Department, Faculty of Medicine, Zagazig, Egypt.

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ABSTRACT

Background: Interleukin 23 (IL 23) and Interleukin 17 (IL 17) are two vital cytokines encountered in cutaneous and non-cutaneous immune-mediated inflammatory conditions and also share in cancer development. Basal cell carcinoma (BCC) is a well-known skin tumor with a high incidence and locally invasive effect but rare metastatic potential and a low mortality rate. The aim of study was to investigate the association of IL 23 and IL 17 in BCC by immunohistochemical evaluation of both

Methods: Based on a study conducted by us, twenty patients with BCC previously treated with intralesional methotrexate were involved in this study. Both interleukins were evaluated in tumor cells and inflammatory cells pre and post treatment, and expression was compared with treatment response.

Resuts: Overexpression of IL 23 and IL 17 was observed in BCC, moreover, both were decreased post treatment with a significant difference ($p < 0.001$ for both). The relationship between expression and treatment response showed more decreased expression with better response, significantly within tumor cells expressing IL 17 ($p < 0.005$).

Conclusions: IL 23 and IL 17 could be considered as main cytokines sharing in BCC progression and anti-IL 23 and anti-IL 17 therapies might be beneficial.

Key Words: basal cell carcinoma, interleukin 17, interleukin 23, methotrexate .

INTRODUCTION:

Interleukin 23 (IL 23) is a cytokine that belongs to the IL 12 family. It has two subunits: IL 23p19 and IL 12/23p40 [1]. IL 23 is produced by activated macrophages and dendritic cells in many peripheral tissues, such as skin in psoriasis, the intestinal mucosa in inflammatory bowel disease and the joints in rheumatoid arthritis, emphasizing IL 23's critical role in the development of a wide range of autoimmune disorders. [2-4].Describing how the immune system is activated by IL 23, activation of T helper 17 (Th17) cells, a unique subpopulation of γδ T cells, subtypes of natural killer T cells, and innate lymphoid cells are the most well-known responses to IL 23 activation. When Th17 cells are stimulated by IL 23 and certain cytokines, they produce interleukin 17A, also known as interleukin 17 (IL 17) [5,6]. IL 17, IL 23, as well as IL 22 are IL 23/Th17-related cytokines that have been demonstrated to participate in the pathogenesis of skin immune-mediated inflammatory conditions[7]. Aside from their role in inflammation and autoimmunity, Th17 lymphocytes and IL-17 are now thought to play a role in cancer progression as active pro and antitumor factors[8]. encouraging the generation of cancer -initiating cells [9]. and repressing the cytotoxic activity and tumor regulatory T cells[10]. IL 17 has been linked to various cancers as breast cancer, and colorectal cancer[11,12]. The most prevalent skin cancer is basal cell carcinoma (BCC), which is also considered the most common cancer in people with fair skin [11]. BCCs are epidermal skin tumors that develop slowly, are locally destructive, and have malignant behavior, but are not considered fatal[12]. The interaction of

inherent biological variables and exogenous factors including immunologic mechanisms raises the risk of BCC development, [13,14]. as IL 23/Th17-related cytokines and IFN- γ are encountered in BCC development and inflammation following imiquimod treatment or photodynamic therapy[15]. Moreover, serum IL 17 levels were raised in BCC[16].

Surgical excision of BCC is the standard treatment. However, in some circumstances, surgery may not be the perfect decision. On the other hand, many surgical and non-surgical interventions have been used trying to accomplish total clearance without recurrence at follow-up and obtain acceptable cosmetic results with minimum discomfort or side effects pre or postoperatively, which is the ideal result[17].Among non-surgical interventions, methotrexate (MTX) has been recently used in non-melanoma skin cancer as intralesional MTX appears to be a safe and effective therapy for keratinocyte tumors, particularly keratoacanthoma but to a limited extent regarding squamous cell carcinoma[18]. In a prior study conducted by us, we discovered that intralesional MTX can be utilized as a successful and secure therapeutic option for BCC that can be used in conjunction with surgery[19].In continuation to our previous work, we hypothesized that IL 23 and IL 17 immunohistochemical evaluation before and after intralesional MTX treatment in BCC cases and their relationship with treatment response will further clear the evidence of these interleukins' contribution to BCC pathogenesis.

METHODS

This retrospective study was held in Pathology Department and Dermatology, Venereology and Andrology Department, Faculty of Medicine, Zagazig University Hospitals. Zagazig University's Institutional Review Board approved this study (ZU.IRP.4690.6-6-2018) which included 20 patients of BCC subjected to intralesional MTX treatment who were involved in our prior study and evaluated for treatment response clinically and pathologically, which was divided into 5 no, 7 partial, and 8 complete responses [19]. All study participants provided written informed consent and this study follows the Code of Ethics of the World Medical Association (Declaration of Helsinki) for humans' studies. Patients were 10 males and 10 females, with an age ranging from 43 -75 years. The clinical variants of BCC cases were 9 nodulo-ulcerative, 4 superficial, 3 pigmented, 2 nodular, and 2 morphea-like variants. Paraffin blocks of all patients before and after intralesional MTX treatment were previously evaluated for hematoxylin and eosin staining and categorized into 10 nodular, 6 morphea like, 3 adenoid, and 1 infundibulocystic types. In this study, other sections were cut for IL 23 and IL 17 immunohistochemical expression.

To inhibit endogenous peroxidase, serial sections from paraffin blocks were deparaffinized and rehydrated before being placed in 3 percent hydrogen peroxide and subcultured for 10 minutes. Then, the sections were washed thoroughly with phosphate-buffered saline and incubated overnight at 4˚C with primary antibodies to polyclonal IL 17A (ThermoFisher Scientific, Catalog # PA1-84183) and purified monoclonal anti-human IL 23 (p19) (BioLegend Way, San Diego, Catalog# 511201). Sections were coloured with 3,3'-diaminobenzidine and treated with Mayer's hematoxylin.

IL 23 and IL 17 were both detected as cytoplasmic stains. Both tumor and inflammatory cells were tested for positivity. The intensity and percentage of stained cells were combined into a single score. The positive cell percentage was calculated as 100 cells / 4 HPF. The immunohistochemical expression of IL 23 and IL 17 was assessed semi-quantitatively by the percentage of positively stained cells and scored as (0, 5%; 1, 5-25%; 2, 26-50%; and 3, 51-75 percent; 4, >76 percent). The intensity was classified as follows: (0: no expression; 1: weak; 2: moderate; and 3: strong). A combined score calculated by the sum of both percentage and intensity scores was considered, producing an overall score of 0 to 7, where (- or negative) is 0 or 1 point; (1+ or weak positive) is 2-3 points; (2+ or moderate positive) is 4-5 points; and (3+ or strong positive) is 6-7 points. [20]

A digital camera (MU1000A, Amscope, USA) and a binocular microscope (MC30, Micros, Austria) were used to capture IL 23 and IL 17 immunohistochemical stains.

Statistical analysis

Data were tested and evaluated using SPSS (Microsoft Excel software. Inc., Chicago, Illinois, USA), version 23 for data processing. Qualitative variables were stated as number and percentage, whereas quantitative data were stated as mean ±SD. The comparison was done using Wilcoxon signed-rank test to check paired data before and after a time and $\chi 2$ test to explain the association between row and column variables. P value of < 0.05 indicates significant results.

RESULTS

IL 23 and IL 17 expression were observed in both inflammatory cells surrounding tumor cells and in malignant tumor cells. In pre-treatment evaluation, IL 23 was completely positive in all cases, ranging from moderate (45%) to strong positivity (55%) in tumor cells and moderate (65%) to strong positivity

(35%) in inflammatory cells. On the other hand, IL 17 also showed complete positivity throughout all BCC ranging from weak (10%), moderate (45%) to strong positivity (45%) in both tumor and inflammatory cells (Figure 1, Table 1). After intralesional MTX treatment, IL 23 expression in both tumor and inflammatory cells showed a highly significant statistical decrease ($p < 0.001$ for both) compared to before, with a percentage of change of -57.5% for tumor cells and -52.5% for inflammatory cells, as IL 23 showed a change in expression into negative (5%), weak positive (85%) and 10% of moderate positivity in tumor cells and negative (10%), weak positive (70%) and 20% of moderate positivity in inflammatory cells without strong positivity post-treatment in any case. Furthermore, a statistically significant decrease in IL 17 expression in both tumor cells and inflammatory cells has been observed ($p < 0.001$ for both) compared to before treatment, with a percentage of change a -68.33% for tumor cells and -59.17% for inflammatory cells, as IL 17 showed less expression than before MTX treatment to be negative in 30%, weakly positive in 60%, and 10% with moderate positivity in tumor cells and 20% negative, 55% weak positive, and 25% moderate positivity in inflammatory cells with no strong positivity in any case after treatment (Figure 1, Table 1).In relation to the treatment response of patients towards MTX treatment, those patients who showed a partial or complete response to MTX treatment showed a noticeable decrease in IL 23 and IL 17 expression after treatment. We observed a statistically significant decrease ($P<0.005$) in IL 17 expression in tumor cells after treatment in responder patients. However, decrease in inflammatory cells was non-significant. Moreover, the decrease of IL 23 percentage in tumor and inflammatory cells after treatment was associated with an increased response among the studied cases but without statistical significance (Table 2).

Table (1) : comparative results regarding Vas back score and follow up Table1: age and sex distribution among studied group (N=24)

		Age/ years	
Median (Range)		**11.0 (10-18)**	
		N	**%**
Gender	**Female**	**10**	**41.7**
	Male	**14**	**58.3**
	Total	**24**	**100.0**

Table2: bladder sensation distribution among studied group (N=24)

		N	**%**
Bladder sensation	**Intact**	**10**	**41.7**
	Decrease	**2**	**8.3**
	Increase	**12**	**50.0**
	Total	**24**	**100.0**

Table 3: Detruser stability and Bladder compliance distribution among studied group (N=24)

		N	%
Detruser stability	No uninhibited detruser contractions	7	29.2
	Phasic uninhibited detruser contractions	17	70.8
Bladder compliance	Decrease	7	29.2
	Normal	17	70.8
	Total	24	100.0

Table 4: Post Voiding volume distribution among studied group (N=24)

		N	%
Post voiding volume	NIL	21	87.5
	20	1	4.2
	40	1	4.2
	150	1	4.2
	Total	24	100.0

Table 5: diagnosis distribution among studied group (N=24)

		N	%
Diagnosis	Normal	6	25.0
	Hypo contractile bladder	2	8.3
	Idiopathic detruser over activity	16	66.7
Low compliance	No	22	91.7
	Yes	2	8.3
	Total	24	100.0

Figure (1): Laborie triton.

Figure (2) : Uroflowmetry.

DISCUSSION

Basal cell carcinoma (BCC) is the most common cutaneous malignant tumor tightly associated with several risk factors that have been shown to participate in BCC evolution. Environmental factors mainly sun exposure, ionizing radiation, immunosuppression, and association with genetic mutations and genetic syndromes, most commonly nevoid basal cell carcinoma, are a heterogenous group of various contributors[21]. Although surgical excision of BCC is the preferable choice for treatment, other factors to consider when choosing a treatment as the location, like the face, as maintaining function and cosmesis areas is critical. So, several surgical and pharmacologic options are now provided beside surgical excision, e.g., Mohs surgery, electrodesiccation and curettage for surgical options, and topical 5-fluorouracil or imiquimod, intralesional injection, and photodynamic therapy as pharmacologic options[17]. Methotrexate (MTX) is considered an extensively used therapeutic option for treating cancers that inhibits folic acid reductase enzyme[22]. To our knowledge, only two studies have used MTX as a treatment for BCC, with no observed improvement in the studied cases for both studies. This might be related to the minimal number of patients with BCC, where Balighi et al. involved eleven cases of BCC, while Gualdi et al. involved only two cases[23,24]. However, our preceding study showed variable responses up to a complete response using intralesional MTX[19].

IL 23 has a critical role in producing T-cell-mediated responses. Following IL 23 activation, specific T cells and innate lymphoid cells produce IL 17, which includes six subtypes from IL 17A to IL 17F[25,26]. It is a key promoter of immunogenic diseases. The immunotherapies targeting IL 23 and IL 17 in different immune-mediated conditions verify these cytokines' essential roles in various diseases. For instance, Ustekinumab is an IL 23 and IL 12 inhibitor that

binds to the p40 subunit, and Brodalumab and Ixekizumab act as anti-IL 17 agents in the treatment of several immune-mediated inflammatory diseases[27]. All variants of BCC involved in this work showed increased IL 23 and IL 17 expression in tumor cells and surrounding inflammatory cells, suggesting their essential role in BCC pathogenesis. After using intralesional MTX, the two cytokines showed regression in their expression, significantly with IL 17. Nardinocchi et al. demonstrated high IL 17 and IL 22 in BCC, implying that these cytokines aided tumor growth. [28] Also, IL 23 and IL 17 with other cytokines have been expressed in BCC using both immunohistochemistry and quantitative Real Time PCR techniques, and their changes with therapy[15]. Therefore, based on these interleukins' overexpression in BCC and their regression after intralesional MTX treatment, MTX inhibits both interleukins in BCC, resulting in a better response, and possible therapeutic benefit could be achieved by using antagonists for IL 23 and IL 17.

CONCLUSIONS

In BCC, IL 23 and IL 17 levels are overexpressed, notably in inflammatory cells and even more so in tumor cells. The role of these cytokines in BCC proliferation and development was verified by their overexpression in BCC and clinical improvement with decreased cytokine expression after intralesional MTX

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CONFLICTS OF INTEREST
There are no conflicts of interest

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Table (S1): Comparison between urodynamically abnormal and normal patients.

			Abnormal	Normal	t/ X^2	P
Age			**12.0±2.08**	**11.66±1.63**	**0.355**	**0.726**
Bladder capacity			**225.83±82.96**	**265.0±49.29**	**1.084**	**0.290**
Pdet Q max			**51.27±8.32**	**57.83±4.02**	**1.839**	**0.079**
Q max flow			**14.94±2.33**	**15.16±1.16**	**0.225**	**0.824**
Voiding volume			**214.16±61.21**	**265.0±49.29**	**1.836**	**0.080**
Gender	**Female**	**N**	**7**	**3**		
		%	**38.9%**	**50.0%**		
	Male	**N**	**11**	**3**	**0.229**	**0.633**
		%	**61.1%**	**50.0%**		
Bladder sensation	**Intact**	**N**	**4**	**6**		
		%	**22.2%**	**100.0%**		
	Decrease	**N**	**2**	**0**	**11.20**	**0.004***
		%	**11.1%**	**0.0%**		
	Increase	**N**	**12**	**0**		
		%	**66.7%**	**0.0%**		
Detruser stability	**No uninhibited detruser contractions**	**N**	**1**	**6**		
		%	**5.6%**	**100.0%**		
	Phasic uninhibited detruser contractions	**N**	**17**	**0**	**19.42**	**0.00****
		%	**94.4%**	**0.0%**		
Bladder compliance	**Decrease**	**N**	**7**	**0**		
		%	**38.9%**	**0.0%**		
	Normal	**N**	**11**	**6**	**3.29**	**0.07**

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