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ORIGINAL ARTICLE

Human Papilloma Virus Genotypes and Induced Protein-10 as Predictors for the Clinical Response to *Candida* Antigen Immunotherapy of Warts

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ABSTRACT

Background: *Warts constitute the commonest cutaneous manifestation of human papillomavirus (HPV) infection. Intralesional* Candida *antigen immunotherapy is used for wart treatment especially with resistant cases.* ***Objective:*** *To detect the association between different HPV genotypes, level of induced protein 10 (IP-10) and the clinical response to Candida antigen immunotherapy.* ***Methodology****: A cross-section study was conducted on 57 patients with resistant warts. All patients were injected with Candida antigen intralesionally at 2-weeks intervals for six treatment sessions. Clinical response was evaluated after 6 sessions. HPV genotyping was performed using real time PCR. Whole blood from patients was incubated with Candida antigen and IP-10 level was measured by ELISA.* ***Results****: Among the 57 injected patients, 31 (54.4%), 18 (31.6%), 8 (14%) show complete, partial and no response respectively. The most frequently detected genotype was HPV-39 (7.1 %) from HPV positive samples. Viral genotype had no significant relation (P=0.305) with patients' clinical response. Statistically significant different IP-10 levels (P<0.001) were detected with different clinical responses.* ***In conclusion:*** *HPV genotype has no significant relation with the clinical response to Candida antigen immunotherapy in wart patients. IP-10 level is an excellent predictive factor for the immune response and hence for the clinical response in those patients.*

INTRODUCTION

Human papillomaviruses (HPVs) are small circular double-stranded DNA viruses. The HPV virion is 55- 60 nm in diameter. The capsid lacks an envelope, making HPV very stable, infectious for years and resistant to many therapeutic agents[1]. Warts are the commonest manifestation of HPV infection; they can be classified into cutaneous and mucosal types. The most frequent HPV types in cutaneous infections are 1, 2, 3, 4, 27, and 57.In mucosal types, the most commonly found high-risk types of HPV are HPV16, 18, 31, 33, 52, and 58. On the other hand, HPV types 6, 11 and 35 are the most commonly found low-risk types [2].

Warts are primarily treated by destructive therapies such as cryotherapy, intralesional chemotherapeutic agents such as bleomycin, oral immune modulators like cimetidine or even oral antivirals like cidofovir. Response to any of these treatment options is highly variable and patient dependent. Moreover, even after patient has responded to any of these treatment options, there are chances of relapse [3].

Intralesional antigen immunotherapy represents a promising therapeutic approach for the treatment of different types of warts, particularly the multiple and recalcitrant variants. Different types of antigens have been utilized, either as a single antigen or as a combination of antigens [4, 5]. Previous studies have documented the efficacy of intralesional *Candida* antigen immunotherapy in wart resolution especially with resistant warts[6] .

The mode of action of intralesional immunotherapy is basically related to its ability to induce a strong cell-mediated immune reaction to alter the balance between Th1 and Th2 responses in favor of the former, leading finally to eradication of HPV[4]. *Candida* antigen alone induces Interleukin-12 (IL- 12) secretion by Langerhans cells *in vitro* when it contacted with it. IL-12 stimulates Thl cell subpopulation, so it seemed possible that it would stimulate proliferation of Thl cells producing IFN-γ. Moreover, intralesional antigen injection further induces production of induced protein- 10 (IP-10) in asignificant level[7].

In spite of this, variable clinical responses have been documented in previous studies [8, 9],which is an issue remains to be clarified.

The aim of this work is to study the different HPV genotypes and immune response to *Candida* antigen as predictors for clinical response to intralesional *Candida* antigen immunotherapy of wart.

METHODOLOGY

A cross-sectional study was carried out in Microbiology & Immunology Department, Faculty of Medicine, Zagazig University and Outpatient Clinic of Dermatology, Venereology and Andrology Department at Zagazig University Hospitals.The study was approved by the Institutional Reviewer Board (IRB), Faculty of Medicine, Zagazig University.

Patients

This study was conducted on 57 patients who had resistant or recurrent warts. They were recruited from the Outpatient Clinic of Dermatology, Venereology and Andrology Department at Zagazig University Hospitals. A written informed consent was taken from all patients before the start of the study. Each patient was primarily subjected to clinical history taking and dermatological examination.

Shave biopsy was taken from wart lesion, as a portion of the lesion above the level of the surrounding skin, in all participants. In addition, two ml blood were collected in heparin containing tubes.

Intralesional *Candida* antigen injection:

All patients were directly injected with 0.1 ml of 1/1000 solution of *C.albicans* antigen; *C.albicans* 1:20w/v 10 ml vial (Allergy Laboratories, INC. Oklahoma City, USA.). The antigen was injected in each lesion, if possible. In coalesced or tiny lesions only the mother wart was injected. Injections were done at 2-week intervals until for six treatment sessions [10]. Overall clinical response was interpreted as follows; Complete: 100% warts were no longer visible, partial: 50–99% improvement and no response: <50% improvement. Interpretation was dependant on the decrease in size of the injected wart as well as the decrease in size and number of distant warts[11].

DNA extraction from wart biopsy samples:

Using (DNA Technology PREP-NA-PLUS, Russia) was performed following the manufacturer instructions.

PCR detecting β-Globin gene:

DNA integrity was assessed by SYBR Green based real time PCR amplification of a 268 bp segment of the human β-Globin housekeeping gene[12], using Super Real Pre Mix Plus kit and the following Primers (GH20):5'GAAGAGCCAAGGACAGGTAC3'.and(PC04):5'CAACTTCATCCACGTTCACC3'.Twenty five microliters of reaction solutions were prepared (5µlDNA template and 0.75µl (10µM) of each primer) and amplification was carried out in real time thermal cycler (Mx3000p QPCR). Thermal profile was performed as follows: Initial denaturation at 95°C for 10 min followed by 50 cycles of denaturation at 95°C for 10 sec and annealing/ extension for one min at 60°C.

Detection of HPV DNA:

β-Globin positive samples were subjected to conventional PCR that amplifies 450 bp product for L1 ORF of HPV [13], using Thermo Scientific-Dream Taq-Green PCR Master Mix and the following primers (MY09): 5' CGTCCMARRGGAWACTGATC 3' and (MY11): 5' GCMCAGGGWCATAAYAATGG 3'.Twenty five microliters of reaction solutions were prepared(2µl DNA template and 1µl (10µM) of each primer) and amplification was carriedout in a DNA thermal cycler (Biometra, Germany). PCR was performed as follows: Initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing for 30 sec at 45°C, extension at 72°C for one min. The final extension step was extended to 10 min at 72°C. Amplified products were visualized on 2% agarose gel under UV light.

Genotyping of HPV:

Quantitative REAL-TIME PCR Kit (DNA Technology-HPV QUANT-21): is an *in vitro* DNA test, which is intended for the specific identification and quantification of low-risk (HPV 6, 11, 44) and high-risk (HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) HPV in human biological samples. The strips were loaded then set to real time PCR instrument (DNA Technology, Russia) and the program was started. Real-time PCR software "HPV_quant-21" was downloaded from «HPV_QUANT_en.ini».

Whole blood culture:

All steps were carried out inside the biosafety cabinet under complete aseptic conditions : 500 µl of whole heparinized blood obtained from patients was cultured in 0.5 ml of RPMI-1640 containing 10% fetal calf serum, 2% penicillin-streptomycin (P/S) in 3 sterile falcon tubes. One tube of diluted whole blood was incubated with 2µl of *Candida* antigen. The second tube of diluted whole blood was incubated with 2µl of Phytohemagglutinin as a positive control. The third tube of diluted whole blood was incubated with 2µl of RPMI-1640 as a negative control. All tubes were put in a sterile holder and incubated for 48 hours at 37˚C in humidified 5% CO2 [14]. After the incubation, the supernatant was collected and stored at -20˚ C for detection of IP-10 by ELISA.

Detecting the level of IP-10:

This was performed using ELISA kit (BIOMATIK-EKU05109) which is a sandwich enzyme immunoassay for *in vitro* quantitative measurement of IP-10.It was measured in cell culture supernates. Steps were done following the manufacturer instructions. Then optical density of each sample was measured at 450nm immediately by ELISA reader **(Stat Fax)**.

Statistical Analysis:

Data were checked, entered and analyzed using Statistical Package for the Social Sciences software SPSS version 25 (SPSS Inc., Chicago, IL) used in Windows 10 for data processing and statistic. Data were expressed as number and percentage for qualitative variables and mean ± standard deviation for quantitative one. The comparison was done using Analysis of variance (ANOVA) test for comparison of means of

multiple independent groups of normally distributed data and Chi- square test (X^2) used to find the association between row and column variables. Receiver Operating Characteristic (ROC) curve is a graphical plot of sensitivity against 1-specificity that illustrates the diagnostic ability of a test. Sensitivity, specificity, predictive values and accuracy were calculated in relation to the gold standard. Results were considered statistically significant when *P* (probability) values were equal to or less than 0.05 at confidence interval (CI) 95%.

RESULTS

This study was performed on 57 wart patients including 24 males and 33 females with their ages ranging from 4- 56 years. Demographic data and clinical characteristics of the studied patients are summarized in **Table (1)**.

Table 1: Demographic data and clinical characteristics of the studied patients

Characteristics	**No.=57**	
Age (years)		
Mean ± SD (Range)	25.5±12.9 (4-56)	
Age groups	**No.**	**%**
0-10	10	17.5
>10-20	8	14.0
>20-30	**23**	**40.4**
>30-40	10	17.5
>40-50	3	5.3
>50	3	5.3
Sex	**No.**	**%**
Male	24	42.1
Female	33	57.9
Wart duration (months)	**No.**	**%**
2-6	**32**	**56.1**
> 6-12	12	21.1
>12-24	9	15.8
>24	4	7.0
Location of wart	**No.**	**%**
Single site	49	86.0
➢ Anogenital	3	5.3
➢ Face	3	5.3
➢ Leg	5	8.8
➢ Palmar	6	10.5
➢ Periangual	6	10.5
➢ **Planter**	**26**	**45.6**
More than one site	8	14.0
➢ Face & palmar	4	7.0
➢ Leg & palmar	1	1.8
➢ Planter & periangual	3	5.3

Table (2) shows the therapeutic response to *Candida* antigen immunotherapy in injected and non injected warts. Regarding the overall clinical response of injected patients, 31 (54.4%) injected patients showed complete clearance of wart lesions Figures (1 a,b), (2 a,b) and (3 a,b), 18 patients (31.6 %) had partial response with decrease in the size of their warts Figure (4 a,b), while 8 patients (14%) showed no change in the size of their warts following the injections.

Table 2: Therapeutic response to *Candida* antigen injection among the studied patients.

Variable	**No.**	**%**
Injected warts	**57**	
Complete response	33	57.8
Partial response	14	24.6
No response	10	17.6
Non injected (distant) warts	**28**	
Complete response	18	64.3
Partial response	4	14.3
No response	6	21.4
Overall response *	**57**	
Complete response	31	54.4
Partial response	18	31.6
No response	8	14.0

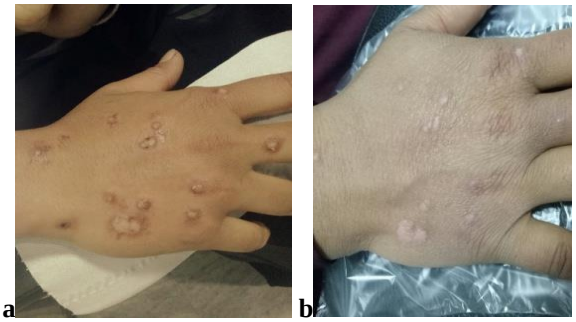

Fig. 1: Multiple palmar warts at the dorsum of the right hand: a) Before intralesional *Candida* Ag immunotherapy. b) Complete response after 6 sessions.

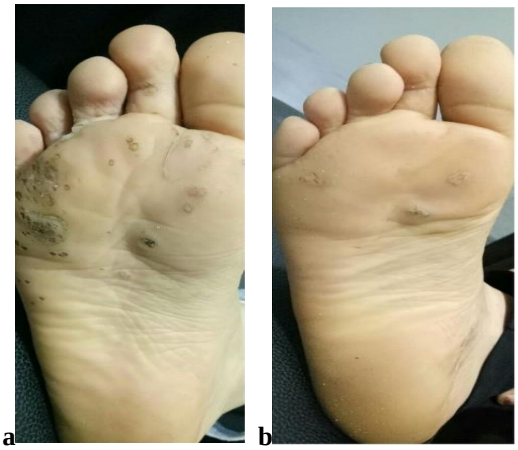

Fig. 2: Multiple planter warts at the sole of the right foot: a) Before intralesional *Candida* Ag immunotherapy. b) Complete response after 6 sessions.

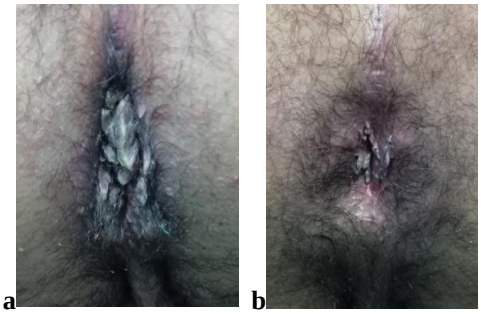

Fig. 3: Multiple anogenital warts coalesced and form mass around anus: a) Before intralesional *Candida* Ag immunotherapy. b) Complete response after 6 sessions.

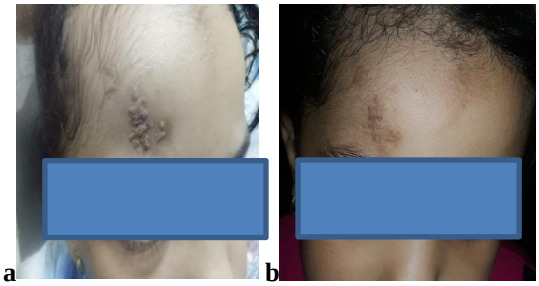

Fig. 4: Multiple common warts at face: a) Before intralesional *Candida* Ag immunotherapy. b) Partial response after 6 sessions (the noticed pigmentation is caused by previous salicylic acid therapy).

The most frequent adverse effect was pain which was recorded in all patients, erythema in (n=9, 15.8%), edema (n=5, 8.8%), flu-like symptoms (n=6, 10.7%), fever (n=3, 5.3%) and the least frequent was lymphadenopathy, recorded in only (n=2, 3.5 %).

β-Globin gene was considered as a control (housekeeping) gene and had been detected by real time PCR in all 100% (n=57) biopsy samples.HPV DNA was detected by conventional PCR in 46.4% (n=26) of all biopsy samples while 53.6%(n=31) were negative Figure(5).

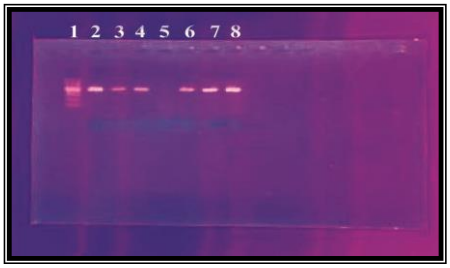

Fig. 5: Agarose gel electrophoresis showing the results of conventional PCR detection of HPV DNA in biopsy samples: **Lane 1:** 100 bp DNA ladder. **Lane 2,3,4,6,7 and 8:** 450 bp band +ve for L1 ORF of HPV.**Lane 5:** No band (–ve for L1 ORFof HPV)

The most common detected HPV genotype among HPV positive samples in the current study was HPV- 39 (n=4, 7.1 % of cases), other genotypes were detected as HPV-16, HPV-31 and HPV-35 (n=3, 5.4%), HPV-26, HPV-52, HPV-53 and HPV-66 (n=2, 3.6%), HPV-18 (n=1, 1.8%) and other genotypes (n=4, 7.1 %). No statistically significant difference between age, sex, duration and location of warts, previous therapies, adverse effects and overall clinical response is found. Moreover, there is no statistically significant difference between the detection of HPV DNA nor the different HPV genotypes detected in wart tissue biopsies and overall clinical response (*P*=0.838& *P*=0.305 respectively).

A significantly higher levels of IP-10 (P<0.001)were recorded in the supernatant of whole blood in those with complete response (Mean ± SD 1140.1 ± 71.8×10 pg/ml) compared to those with partial response (Mean ± SD 839.7 ± 123.8×10 pg/ml) and those with no response (Mean ± SD 528.1 ± 58.1×10pg/ml) **Figure(6)**.

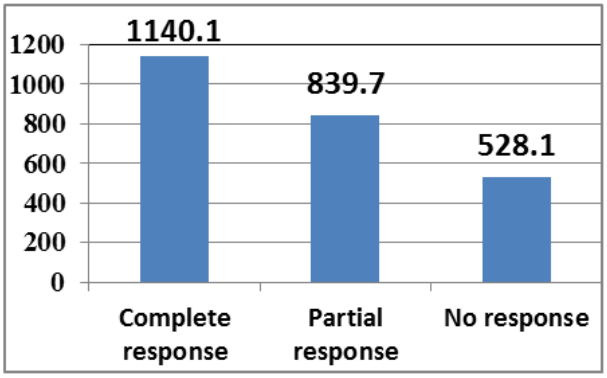

Fig. 6: Level of IP-10 (×10 pg/ml) in patients with complete, partial and no responses. Detected in supernatant of whole blood culture.

The cut off value of IP-10 was set at 604 ×10 pg/ml. At this level the area under the curve equals one with 95% CI of 1-1. This indicates that patients with levels higher than 604 ×10 pg/ml can be considered as responders while those with levels lower than 604 ×10 pg/ml are considered non responders Table (3) and Figure (7)with 100% sensitivity, specificity, +ve predictive value, -ve predictive value and accuracy.

Table 3: Cut off value of IP-10 detected by ELISA among the studied group.

ELISA IP-10 (pg/ml)×10	Overall Clinical Response		Cut off value	Area under the curve	95% Confidence interval	*P*.value
	+	-				
≤ 604	0	8	604	1	1-1	<0.001**
> 604	49	0				

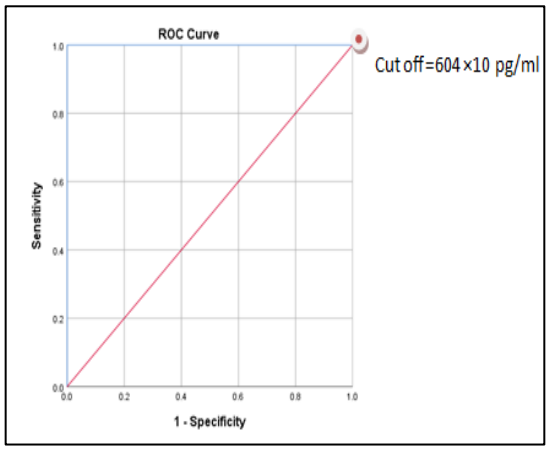

Fig .7: ROC curve demonstrating the validity of measurement of IP-10 in supernatant of whole blood culture with *Candida* antigen in comparison with the overall clinical response (gold standard) among the studied group.

DISCUSSION

In present study, we tried to find out if HPV genotype has any relation with the clinical response to *Candida* antigen immunotherapy in recalcitrant (resistant) warts. Furthermore, we attempted to use the level of IP-10 as an immunological predictor for response in those patients. Intralesional injection of *Candida* antigen was conducted in 57 patients suffering from recalcitrant (resistant) warts. The mean age of the studied group was 25.5 years ± 12.9. This comes nearly similar to the result of Singh et al. [15] who found that the mean age of the patients in his study was 25.98 years and with Majid and Imran, [16] who found that the mean age in their studied group was 24.3 years.

Females in this study represented (n=33, 57.9%) while males were (n=24,42.1%). Also, Nofal et al. [9] in a previous Egyptian study reported more females in his study (n=33, 61.1%) compared to (n=21, 38.9%) males. Horn et al. [17] had similar sex distribution as females in his study were (n=31, 57.4 %) and males were (n=23, 42.6 %). The obvious higher ratio of female patients which is reported in Egyptian studies may be attributed to their cooperation and agreement to participate in medical studies and perhaps to their higher seek for medical advice to treat warts which form a cosmetic problem in our community.

The overall response to *Candida* antigen immunotherapy in the present study comes lower than Nofal et al., [9], who followed the same regimen as that of ours. They reported complete response in 61.1% of the studied patients (n=33), partial response in 29.6% (n=16) and no response in 9.3% (n=5). The lower percentage among responders in our study, can be returned to the difference in the criteria of included patients where all patients in our study had recalcitrant warts compared to only 13% in Nofal et al., [9] study.

On the other hand, the ratio of the non responders in the present study (17.6%) comes lower than Majid and Imran, [16] who studied resistant and recurrent warts and reported complete response in 56% of their patients (n=19), partial response in 6% (n=2) and no response in 38% (n=13). The different treatment regimen applied in our study; longer duration of immunotherapy and shorter intervals between sessions which were set at 1-weeks intervals (compared to three week in their regimen) for a total of six doses (compared to only three in theirs) may explain this difference.

Pain was reported in all injected patients in our study. Similarly, Majid and Imran, [16] recorded pain in the majority of their studied group. While, Perman et al., [18] reported that pain could be completely abolished with local regional block of the site of the wart with 1% lidocaine prior to injecting *Candida* antigen or with intralesional injection of lidocaine.

HPV DNA was found in the wart biopsy in (n=26, 46.4%), the current study using the primer MY09-11 to detect L1 ORF. In the study carried out by Giannaki et al., [19], HPV DNA was found in 75% of the wart tissue analyzed, using the same primer. On the other hand, Nobre et al., [20] and Chen et al., [21] found HPV DNA only in 25% and 16.7% of cases, respectively, using the same primer but in cervical samples using cervical brush. This clearly indicates that the sensitivity of MY09-11 primer differs with different tissue specimens. Still the lower ratio of wart tissues yielding viral DNA in our study necessitates further explanation. The biopsy tissue itself which might have been obtained in a superficial way may contribute to this finding. Moreover, the presence of some sort of PCR inhibitors in the biopsy tissue, probably resulting from previous wart therapy, could be another factor.

The most common detected HPV genotype among HPV positive samples in the current study was HPV- 39 (n=4, 7.1 % of cases). Different HPV genotypes have been reported in different previous studies. HPV-27 was the most frequent type identified by Sasagawa and Mitsuishi, [22] which was detected in 44% of cutaneous wart samples from Japanese patients. On the other hand,

Giannaki et al., [19] reported HPV- 57 as the most frequently observed type (43.1%) in cutaneous warts of Greek children. In American subjects, Horn et al., [17] found that among 146 subjects with warts, types 2, 27, or 57 were the most frequent, being found in 120 American subjects. This strongly points to the different HPV genotypes distribution in different geographical localities.

The present study did not show a statistically significant difference (P=0.305) between the different HPV genotypes detected in wart tissue and the overall clinical response in the studied group. The same result was recorded by Horn et al., [17] as there was no significant association between viral type and response to injection (P=0.99). As far as we know, no further studies had addressed this issue. Though we could not fully explain this finding but probably other host factors interact together and might have higher association with patients' response e.g. the efficiency of Langerhans cells as well as other immune cells of the patient, the density of the immune receptors that recognize *Candida* antigen e.g. TLR-4 and the level of immune mediators induced [9]. The paucity of the studies addressing this issue makes it important to go further in more wide studies involving higher number of patients to elucidate the exact effect of viral genotype on *Candida* antigen immunotherapy.

The present study did not record any statistically significant difference between different age groups (P=0.603), patients sex (P=0.375), durations of warts (P=0.826), location of wart (P=0.884), previous therapies (P=0.321) or the adverse effects (P=0.502) of patients and the overall clinical response. This comes in agreement with Khozeimeh et al. [23] where patients' age (P = 0.124), patients' gender (P = 0.642) as well as wart duration before treatment (P = 0.114) did not show any relation to the therapeutic response in the *Candida* immunotherapy group.

In order to find out the relation of the immune mediators released after *Candida* immunotherapy with patients' response, we further assessed the level of IP-10 in whole blood culture of treated patients. Up to our knowledge, this study is the first one to evaluate IP-10 level as a predictor for the immune response to *Candida* antigen immunotherapy in wart patients. Other immune markers have been studied previously. Nofal et al., [9] studied the level of IFN-γ in the supernatant of whole blood culture with *Candida* antigen and revealed a statistically significant increase in IFN-γ levels in responders as compared to non responders. A cut off value was set at 0.89 pg/ml at which the test had a sensitivity of 91.7%, a specificity of 75%, and an accuracy of 85% among the studied patients (P=0.03). The authors suggested that it can be used as a good predictor of the therapeutic response to intralesional injection of *Candida* antigen.

In another study, Horn et al.[17] revealed that responders were more likely to have a positive peripheral blood mononuclear cell (PBMC) proliferation assay result than non-responders (P=0.002) considering this to be a significant predictive factor for clinical response.

Comparing our results with that of Nofal et al. [9] and with Horn et al.[17], it is obvious that the level of IP-10 had performed well and achieved more accurate predictive value for the immune response to intralesional *Candida* antigen immunotherapy and hence to clinical response.

CONCLUSION

Intralesional *Candida* antigen immunotherapy is efficient method for resistant wart therapy. IP-10 level is excellent predictive factor for immune response and hence for clinical response.

Recommendations:

Use of IP-10 level as a predictor for the clinical response to *Candida* antigen immunotherapy of warts especially in resistant cases. Further studies that address other factors that might have more significant relation with clinical response.

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- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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