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CLINICAL RESEARCH

Vaginal Smear *TNF-alpha*, *IL18*, *TLR4*, and *GATA3* mRNA Levels Correlate with Local Inflammation

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Abstract

Background: A molecular approach to estimation of local inflammatory response associated with dysbiotic conditions of the vagina is reported. This approach is based on immune response gene transcription levels measured by qPCR assay.

***Methods and Results*:** qPCR analysis of 24 immune response gene transcripts in vaginal smears was performed for 215 women with vaginitis and 95 healthy controls. The data were sorted by comparison to local inflammatory response profiles assessed by conventional methods. The local inflammatory response in the vagina is found to correlate with *TNF-alpha, IL18, TLR4,* and *GATA3* transcript levels. Sensitivity and specificity of the approach, as validated in accordance with conventional clinical examination results, are 94.9% and 93.7%, respectively.

Conclusions: The noninvasive diagnostic approach to vaginal pathology based solely on its molecular characterization may prove to be clinically relevant.

Keywords: *vaginitis, inflammation, cytokine, mRNA, qPCR.*

List of abbreviations

PCR, polymerase chain reaction; **WBC**, white blood cells; **PPV**, positive predictive value; **NPV**, negative predictive value.

Introduction

Inflammatory diseases of the vagina are very common. The most often cause of them is the presence of pathogenic microbes in mucosal parts of the genital tract. Due to characteristic increase in the number of immune system cells in that region, a conventional clinical approach to local immunity status evaluation, of inflammatory status in particular, includes the counting of white blood cells (WBC) in a vaginal smear. Some shifts in concentrations of immunologically relevant to molecules (cytokines, defensins, immunoglobulins, immune cell receptors etc.) in the smear during inflammatory diseases of the vagina have also been discussed [1,2]. However, the clinical implication of these studies was somehow impeded despite the obvious need for accurate markers describing the state of the local immune system of the vagina.

The current study aimed to develop a method for estimating the local inflammatory response in the vagina on the basis of immune response gene transcription measured in a vaginal smear.

Methods

The exclusion criteria for patients were as follows: positive PCR results for *Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis,* or *Mycoplasma genitalium*. Additional exclusion criteria were pregnancy and the use of antimicrobial medication. Written *informed consent was obtained* from each patient.

Patients (310 females of reproductive age) were divided into two groups of positive (Group 1) and negative (Group 2) for vaginitis. Group 1 consisted of 215 patients with vaginal discharge (77.2%), itching (41.4%), burning (32.6%), odor

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(13.0%), dyspareunia (9.8%), and dysuric disorders (3.3%). During medical examination, the vulvovaginitis signs were edema (40.9%), hyperemia of the vaginal and vulva mucous (69.3%), ulceration of external genitalia (10.7%), labia and anogenital areas maceration (5.6%). The vaginal discharge, pH of vaginal fluid, whiff test and clue cells in a smear was recorded. Usually the abnormally high WBC counts in vaginal smears were characteristic for this group (10 to 60 in sight). Finally the causes of vaginitis of group 1 were: vulvovaginal candidiasis (24%), bacterial vaginosis (14%), aerobe progression (7%), mixed fungal-bacterial infection (28%), HSV1-2, and lack of a detectable etiological agent (23%).

Group 2 consisted of 95 clinically healthy women with no complaints, no signs of inflammation, and the normal WBC counts in vaginal smear (<10). The major vaginal microbiota component was *Lactobacillus*, while the opportunistic pathogenic microbe fraction of the total bacterial mass did not exceed 10%. The exclusion criteria for group 2 were the qPCR results for *Candida* carriage and *Ureaplasma* species more than 10^4 genomes per ml of standard vaginal smear in the transportation medium.

Vaginal smears were collected according to standards of gynecological practice. Immediately after the capture, the probe was immersed in a test tube with 0.5 ml of transportation medium that was thoroughly covered with liquid wrung off the tube wall before the tube was capped. After transportation for 2 to 8 hours at room temperature, the samples were frozen and stored at -20°C for up to 3 months.

Bacterial qPCR tests were performed by a Femoflor-16™ Kit (DNA-Technology JSC, Russia) with total bacteria (by 16S rRNA), *Lactobacillus spp*. and 14 major groups of opportunistic microbial species, including facultative anaerobic and obligate anaerobic forms (*Enterobacterium spp., Streptococcus spp., Staphylococcus spp., Gardnerella vaginalis, Prevotella spp., Porphyromonas spp., Eubacterium spp., Sneathia spp., Leptotrihia spp., Fusobacterium spp., Megasphaera spp., Veilonella spp., Dialister spp., Lachnobacterium spp., Clostridium spp., Mobiluncus spp., Corynebacterium spp., Peptostreptococcus spp., Atopobium vaginae)*, as well as *Candida spp., Ureaplasma spp., Mycoplasma hominis*.

The vaginal smear mRNA qPCR profiling for cytokines (interleukin 1 beta (*IL1β)*, interleukin 6 *(IL6)*, interleukin 8 (*IL8)*, interleukin 10 (*IL10)*, interleukin 12A (*IL12A)*, interleukin 15 (*IL15)*, interleukin 18 (*IL18)*, interleukin 23 (*IL23)*, tumor necrosis factor alpha *(TNF-a)*, interferon gamma *(IFNγ)*, transforming growth factor, beta 1 (*TGFβ1)*, leukemia inhibitory factor *(LIF)*), transcription factors (T-box 21 (*TBX21)*, GATA binding protein 3 (*GATA3)*, RAR-related orphan receptor C *(RORC)*, forkhead box P3 (*FOXP3)*), toll-like receptors (toll-like receptor 2 (*TLR2)*, toll-like receptor 4 (*TLR4)*, toll-like receptor 9 (*TLR9)*) and other immune cell molecular markers (interleukin 2 receptor, alpha *(IL2Rα)*, protein tyrosine phosphatase receptor type C (*CD45)*, CD68 molecule (*CD68)*, CD69 molecule (*CD69)*, granulysin *(GNLY)*) was performed by an mRNA Profiling Kit (DNA Technology JSC, Russia). The reference transcripts were TATA box binding protein, beta-2-microglobulin, glucuronidase-beta with ΔΔCq normalization; Me values in the control group were set to 1. Statistical significance of between-group differences was evaluated by Mann-Whitney test.

Diagnostic criteria were determined by binary logistic regression with a linear function allowing calculating the probability of the inflammatory response (P) by:

$$P = \frac{1}{(1 + e^{-z})} * 100$$

P–probability of the inflammatory response;
*z=k1*x1+k2*x2+.....kn*xn+constant*
x1, x2,... xn – mRNA level;
k1, k2, kn – required coefficients.

Receiver Operator Characteristic Analysis was used to calculate the optimal values for the cut-off threshold with maximal sensitivity and specificity of the method.

Results

mRNA profiling of vaginal smears

The detected mRNA level in vaginal smears is shown in Figure 1. By copy number these transcripts can be divided into three standard groups:

1. High abundant transcripts (10^5 to 10^7 copies per ml of standard vaginal smear in transportation medium): *IL8*, *IL1β*, *IL18*, *TGFβ1*, *CD68*, *GATA3*;

2. Medium abundant transcripts (10^3 to 10^5 copies per ml): *TNFa*, *CD45*, *IL12A*, *IL23*, *TLR2*, *TLR4*, *CD69*, *IL10;*

3. Low abundant transcripts (<10^3 mRNA copies per ml)): *RORC*, *IL15*, *FOXP3*, *LIF*, *TLR9*, *GNLY*, *TBX21*, *IFNγ*, *IL6*, *IL2Rα.*

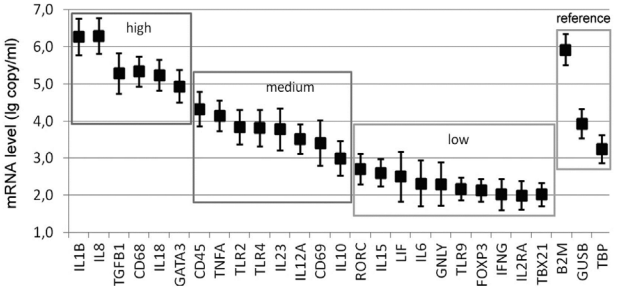

Figure 1. *mRNA species level in vaginal smears (for the total set; n = 310).*

Because the low abundant transcripts are not always detectable in vaginal smears, the genes with low expression were excluded from consideration; thus, only the high and medium abundant transcripts were evaluated as potential markers of the local inflammatory reaction.

We found that mRNA levels of *IL8*, *TLR2*, *TLR4*, *IL1β*, *IL10*, *CD69, CD45*, and *TNF-α* were significantly increased in the vaginitis group as compared to the controls while levels of *IL18*, *GATA3*, *CD68*, *IL12A* and *TGFβ1* were reduced (Table 1).

Table 1.
Statistically significant differences in mRNA levels between the groups

Gene	mRNA level difference (times)	*P*-value
Increased transcription level (in vaginitis)		
IL8	5.7	$p=3.1 \times 10^{-20}$
TLR2	4.2	$p=3.6 \times 10^{-8}$
IL1b	4.0	$p=2.6x\ 10^{-18}$
TLR4	3.9	$p=7.1 \times 10^{-21}$
IL10	3.3	$p=2.3 \times 10^{-17}$
CD69	3.1	$p=5.8 \times 10^{-11}$
CD45	2.7	$p=1.1 \times 10^{-14}$
TNFa	1.4	$p=3.2 \times 10^{-2}$
Decreased transcription level (in vaginitis)		
IL18	9.5	$p=6 \times 10^{-32}$
GATA3	7.9	$p=1.3 \times 10^{-33}$
CD68	4.0	$p=2.4 \times 10^{-23}$
IL12A	3.2	$p=6.7 \times 10^{-19}$
TGFb1	2.1	$p=6.5 \times 10^{-11}$

Derivation of the algorithm

The analysis was performed in two stages. At the first stage both groups of patients were randomly divided into two subgroups (training and validation samples). Training samples consisted of 115 vaginitis patients and 43 control group patients. Accordingly, 100 vaginitis patients and 52 controls were left for the second, the validation, stage. The ratio of most contrast up- and downregulated transcripts was used to derive the local inflammatory response estimation algorithm by regression analysis. Of all possible ratios, two were selected as the most promising based on the data from the training samples: *TLR4*/*GATA3* and *TNF-α*/*IL18* (Figures 2 and 3).

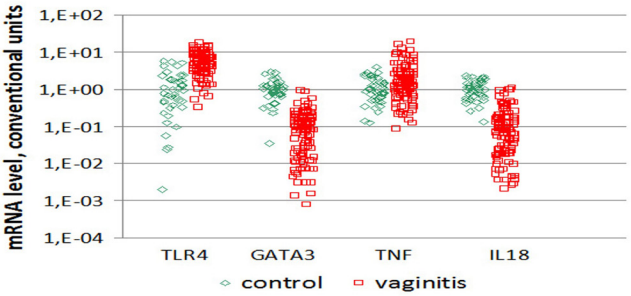

Figure 2. *TLR4, GATA3, TNFα u IL18 transcripts level in vaginal smears of the vaginitis group compared to the controls.*

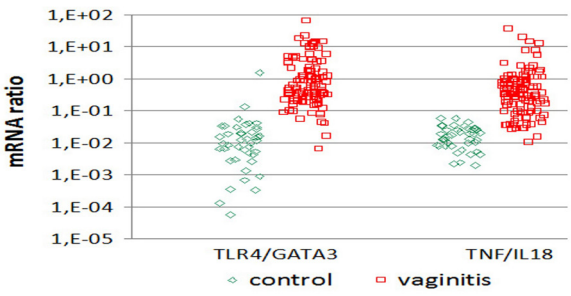

Figure 3. *TLR4/GATA3 and TNFα/IL18 transcripts level ratio in vaginal smears of the vaginitis group compared to the controls (for the training subgroup)*

The equation of the linear function had the form:

$z = 1.4 * \ln([TLR4]/[GATA3]) + 1.3 * \ln([TNF\alpha]/[IL18]) + 7.8$

(TLR4/GATA3) and (TNFa/IL18) meant the ratio of the respective gene expression levels.

The ratio of the respective gene expression levels was calculated by:

$[Gene\ 1]/[Gene\ 2] = 2^{Cq2-Cq1}$

Cq1, and Cq2 are qPCR cycle values (usually Ct or Cp) for corresponding molecular targets.

The optimum value of the cut-off threshold was determined by ROC-analysis. The area under the ROC-curve was AUC=0.989±0.007 (P=3.4x10^{-21}). The probability threshold of inflammation was 57%; probability thresholds above 57% were classified as marking vaginitis. The sensitivity and specificity of the proposed method was 95.7% (110 of 115) and 97.7% (42 of 43). The positive predictive value (PPV) and the negative predictive value (NPV) were 99.1% and 89.4%, respectively. Diagnostic sensitivity and specificity of the proposed method in the threshold for the validation sample was 94% (94 of 100) and 90.4 (47 of 52), respectively (PPV=94.9%, NPV=88.7%) (Table 2).

Table 2.
Diagnostic sensitivity and specificity of the proposed method

Marker	Sensitivity (%)	Specificity (%)	Cutoff
TLR4	85.2	86.0	2.50
GATA3	95.3	92.2	0.28
TNFa	53.0	74.4	1.53
IL18	88.4	94.8	0.49
TLR4/GATA3	95.7	95.3	0.06
TNFa/IL18	94.8	90.7	0.04

The local inflammatory response data for all smears are shown in Fig.4. Of the reunited control group (training plus validation samples) 89 of 95 smears were classified as normal (specificity=93.7%). Reciprocally, in the vaginitis group 204 of 215 smears were classified as showing a local inflammatory response (calculated sensitivity for the reunited training and validation samples was 94.9%).

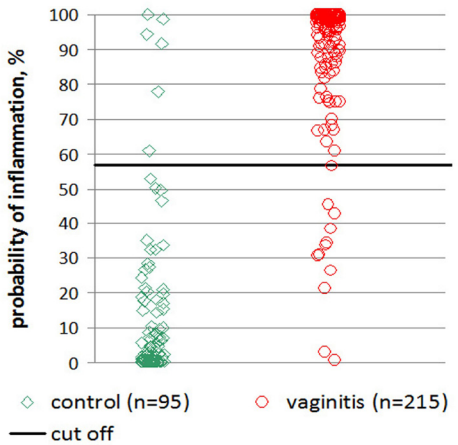

Figure 4. *Calculated individual probabilities of the local inflammatory reaction.*

The Pearson's correlation between the proposed molecular approach and the conventional smear microscopy was r=0.6 (P=3.2x10^{-23}) (Fig. 5).

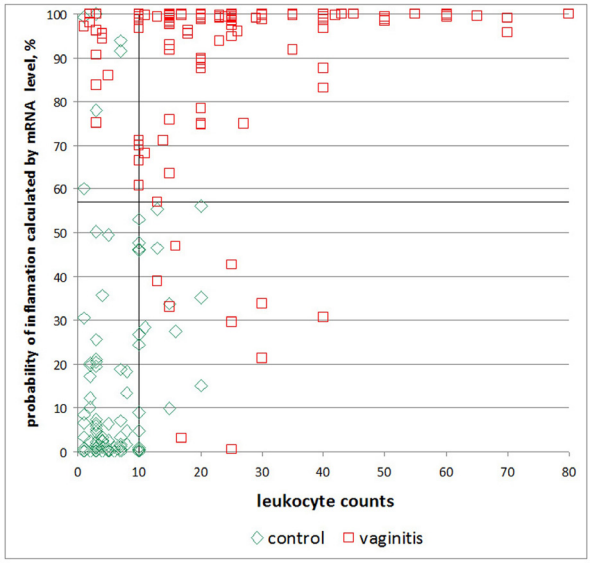

Figure 5. *The correlation between the proposed molecular approach and the conventional smear microscopy*

Discussion

Physicians traditionally diagnose vaginitis using the combination of symptoms, physical examination, pH of vaginal fluid, microscopy, and the whiff test. When combined, these tests have a sensitivity and specificity of 81% and 70%, respectively, for bacterial vaginosis and 84% and 85%, respectively, for vulvovaginal candidiasis (when compared with the DNA probe standard [3]). Thus the sensitivity and specificity of conventional tests are not perfect. At the same time, very precise NAT approaches for detecting local inflammation can significantly improve these parameters.

Detectable changes in the expression level of immune response genes can be used to assess the inflammatory response in dysbiotic conditions of the vagina by introducing a transcriptional profile index. The optimum indexes were observed for pairs *TNF-alpha*/*IL18* and *TLR4*/*GATA3*. Inclusion of the other indexes did not essentially improve the quality of the model. We used the transcripts ratio autonormalized data, so the reference transcripts were not necessary. The shortened list of potential markers significantly simplifies the analysis, allowing the implementation of the approach into routine practice.

The *TNF-alpha* gene is known to be expressed by activated macrophages; it is one of the key markers of inflammatory reactions. Upregulation of TNF-alpha expression was found after Gramnegative bacteria cell wall lipopolysaccharide/TLR4 interaction [4]. The interaction results in activation of macrophages, producing a number of proinflammatory cytokines (IL1, IL6, IL8, IL12, TNFα, and type I interferons (IFNα/β)) [5]. The GATA3 transcription factor is widely known as a positive regulator of the Th2 differentiation program switch [6]. However, this regulation takes place in the regional lymph nodes, rather than locally in the foci of infection. *GATA3* is expressed not only by lymphocyte progenitors but also by some other types of cells; it plays an important role in survival of the mammary luminal epithelial lineage in the breast; it is also required for normal development of skin, adipocytes, and nervous system [7]. The characteristic sign of inflammation and proliferative processes in epidermal cells is a decrease of *GATA3* expression [8]. By parity of reasoning, the decrease of *GATA3* transcription in vaginitis is probably due to the processes of physiological regeneration in vaginal epithelium. Somewhat surprising was the vaginitis-associated reduction for *IL18*, which is a proinflammatory cytokine acting in lymphoid organs in conjunction with IL-12 and participating in the regulation of the Th1 lymphocyte differentiation pathway. However, in local mucosal immunity in the vagina its function may differ.

Conclusions

The proposed approach of molecular verification of the local inflammatory response was developed in tight conjunction with the clinical data analysis and the results of smear microscopy. This approach needs most serious consideration among practitioners and a further clinical approbation. Changes in transcription of immunity related genes in response to dysbiosis (apparently ahead of leukocyte mediated reaction) may be considered the earliest hallmark of inflammation. We believe that the fast noninvasive mRNA detection qPCR tests will become very popular in clinical diagnostics of the local reactions.

Competing interests

The authors declare that they have no competing interests.

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References

1. Campos AC, Murta EF, Michelin MA, Reis C. Evaluation of Cytokines in Endocervical Secretion and Vaginal pH from Women with Bacterial Vaginosis or Human Papillomavirus. ISRN Obstet and Gynecol 2012; 2012:342075.
2. Hickey DK, Patel MV, Fahey JV, Wira CR. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. J Reprod Immunol 2011; 88(2):185-194.
3. Lowe NK, Neal JL, Ryan-Wenger NA. Accuracy of the clinical diagnosis of vaginitis compared with a DNA probe

laboratory standard. Obstet Gynecol 2009;113(1):89-95.
4. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 2011; 34(5):637-50.
5. Ji RC. Macrophages are important mediators of either tumor-or inflammation-induced lymphangiogenesis. Cell Mol Life Sci 2012; 69(6):897-914.
6. Mjösberg J, Bernink J, Peters C, Spits H. Transcriptional control of innate lymphoid cells. Eur J Immunol 2012; 42(8):1916-1923.
7. Ho IC, Pai SY. GATA-3 - not just for Th2 cells anymore. *Cell Mol Immunol* 2007; 4(1):15-29.
8. Racz E, Kurek D, Kant M, Baerveldt EM, Florencia E, Mourits S, et al. GATA3 expression is decreased in psoriasis and during epidermal regeneration; induction by narrow-band UVB and IL-4. PloS One 2011; 6(5):e19806.