A new in vivo confocal microscopy prognostic factor in Acanthamoeba keratitis

Un nouveau facteur pronostique dans la kératite amibienne: apport de la microscopie confocale en vivo

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Summary

Objective. — To identify prognostic factors associated with poor outcomes in Acanthamoeba keratitis (AK).

Methods. — Patients with AK treated at the Beijing Tongren Hospital between January 2008 and January 2012 were included. All patients had corneal scrapings and/or cultures positive for Acanthamoeba and visible cysts on in vivo confocal microscopy (IVCM) examination. Therapeutic penetrating keratoplasty was performed in patients who experienced disease progression or lack of improvement on topical therapy. Patient demographics, clinical characteristics, previous treatment, and IVCM characteristics of the cysts were evaluated. Patients defined as poor outcomes were those requiring therapeutic penetrating keratoplasty. Logistic regression was used to estimate the odd-ratio identifying prognostic factors associated with a poor outcome.

Results. — Twenty-nine eyes of 29 patients were diagnosed as having AK over the study period. IVCM showed clusters and/or chains of Acanthamoeba cysts in 9 patients. Fifteen patients underwent therapeutic penetrating keratoplasty. A late-disease stage on presentation, a deep location of cysts, and clusters or chains of cysts observed with IVCM were significantly associated with a worse outcome. On multivariate analysis, only clusters or chains of cysts observed with IVCM were independently associated with a poor prognosis.

KEYWORDS
Acanthamoeba keratitis; In vivo confocal microscopy; Prognostic factors; Diagnosis

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Conclusion. — The presence of clusters or chains of Acanthamoeba cysts could be a new IVCM criterion allowing the identification of AK patients requiring therapeutic penetrating keratoplasty.
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Introduction

Acanthamoeba keratitis (AK) is a painful and severe corneal infection that can lead to irreversible complications including blindness [1,2]. The clinical diagnosis of AK remains difficult because the clinical presentation is non-specific and often misdiagnosed as viral keratitis [3]. Corneal smears and culture are considered to be the gold standard diagnostic tests for this condition [4]. However, cultures take 2 days to 2 weeks to become positive, with positive rates ranging from 0 to 68% [5,6].

In vivo confocal microscopy (IVCM) provides high-resolution images of ocular surface structures and numerous publications have described the use of IVCM as a non-invasive tool to diagnose AK [4,7–9]. IVCM can show cysts, trophozoites, abnormal corneal nerves, intrastral cavi- ties and infiltration of inflammatory cells in AK [4]. Cysts are observed as round or ovoid hyper-reflective structures with sometimes a double-wall appearance measuring between 10 and 30 µm [4,10]. Because of their particular morphology and reflectivity, cysts can be differentiated from other corneal cells and microorganisms, and are the most identifiable IVCM structures to diagnose AK [4]. IVCM can also be used to measure the depth of Acanthamoeba cysts within the cornea, and to make repeated non-invasive examinations, consequently it has been used to monitor progression and the response to antimicrobial treatment [4,11].

Numerous prognostic factors have been described and evaluated in AK but with conflicting results [12]. Moreover, to our knowledge, there is no study evaluating the relation between IVCM findings and the outcome of AK. Using the need to perform a therapeutic penetrating keratoplasty as the definition of the worse outcome, the purpose of this study was to identify prognostic factors for AK, specifically through IVCM evaluation.

Methods

Patients

We retrospectively analyzed the medical records of patients who were diagnosed with AK between January 2008 and January 2012 at the Beijing Tongren Hospital (Beijing, China). Institutional Board Review approval was granted from the Beijing Tongren Hospital in accordance with the Declaration of Helsinki. All patients referred to our department for suspected infectious keratitis had IVCM examination before smears and microbiologic investigations. In the present study, the diagnosis of AK was based on clinical characteristics, identification of cysts through corneal smears stained with Giemsa and/or positive Acanthamoeba cultures, and visible cysts on IVCM examination.
Clinical evaluation and treatment

Age, gender, known risk factors for AK, previous treatment and diagnosis, interval between the onset of symptoms and the appropriate treatment, clinical findings at presentation and treatment methods data were collected. The severity of disease at presentation was evaluated on the basis of biomicroscopy findings and classified into two stages:

- early-disease stage: epithelial stage, epithelial stage with radial neuritis, anterior stromal disease [2];
- late-disease stage: deep stromal keratitis, ring infiltrate or extracorneal complications (Fig. 1).

Once the diagnosis was established on the basis of positive smears or culture, patients were treated as previously described [12]. Treatment was started with polyhexamethylene biguanide (PHMB) 0.02% and chlorhexidine 0.02% eye drops every hour and then adapted to each individual case [12]. Therapeutic penetrating keratoplasty was performed in patients who had a poor or no response after 3 months of topical therapy, or complications such as corneal perforation, fulminant abscess, uncontrolled intraocular pressure or severe extracorneal complications such as scleritis.

In vivo confocal microscopy

IVCM was performed using the laser scanning Heidelberg Retina Tomograph III Rostock-cornea-module (HRTIII-RCM, Heidelberg Engineering GmbH, Dossenheim, Germany) [10]. First, the area of the corneal infiltrate or corneal ulcer was scanned, followed by the peripheral margins of the lesion. In each area, the focal plane of the microscope was manually adjusted from the superficial tissue to the corneal endothelium in order to scan the whole corneal lesion. Approximately 400 images were acquired for each eye. A positive IVCM examination was defined as the visualization on at least 10 images of typical IVCM features of cysts: round, ovoid or egg-shaped hyper-reflective structures with sometimes a double-wall appearance measuring between 10 and 30 μm [4]. The following parameters were evaluated for each patient: the disposition and depth of cysts (< or ≥ 250 μm) within the cornea.

Ex vivo confocal microscopy (EVCM) analysis of Acanthamoeba cultures

Acanthamoeba from the cornea of one AK patient were cultured on non-nutrient agar overlaid with Escherichia coli. The culture samples were analyzed ex vivo with the HRTIII-RCM and compared to the result of the IVCM examination of the same patient.

Statistical analysis

Patients were divided into two groups based on if they underwent therapeutic penetrating keratoplasty. Patients with poor outcome (cases) were defined as those requiring therapeutic keratoplasty. The risk factors evaluated for statistical analysis were: patient age, gender, delay between onset of symptoms and diagnosis, previous steroid use, disease stage at presentation, IVCM depth and arrangement of cysts. All statistical analyses were performed with SPSS16.0. Univariate analysis was performed using logistic regression to estimate the P-value, odd-ratio (OR) and 95% confidence interval (CI) to determine factors associated with a worse outcome. Multivariable logistic regression was performed using forward stepwise addition for variables that were statistically significant in the univariate analysis. In a separate univariate analysis, variables associated with the arrangement of cysts were analyzed using logistic regression.

Results

Twenty-nine eyes of 29 patients were diagnosed as having AK over the study period. There were 11 women (38%) and 18 men (62%) with a mean age of 44 ± 12 years (range 14 to 65). Twenty-five patients were culture positive for Acanthamoeba (14 in the keratoplasty group) and 4 patients were only smear positive. Risk factors for AK were noted in 21 patients (72%). Sixteen patients had ocular trauma, 3 patients received projection of contaminated water, and 2 patients used soft contact lenses. The mean interval between onset of symptoms and diagnosis was 51 ± 23 days (range 20 to 90). For the 7 cases with early stage disease, this mean interval was 48 ± 15 days (range 30 to 60). All patients had been previously misdiagnosed with viral keratitis (n = 21), bacterial keratitis (n = 13), fungal keratitis (n = 8) or uveitis (n = 2). From those, 11 patients had been previously treated with steroids. At presentation, early-disease stage (stage 1) of AK was observed in 7 (24%) eyes and 22 (76%) eyes had late-disease stage (stage 2).

The IVCM features of Acanthamoeba cysts included double-walled, round- or stellate-shaped highly reflective structures and bright ‘signet ring’ images. A hypo-reflective halo was also observed surrounding some cysts (Fig. 2). IVCM also showed the spatial arrangement of cysts within the corneal tissue. In 20 eyes (69%), the cysts were scattered or sparse with group of cysts ≤ 3 cysts. In 9 eyes (31%), the cysts appeared as chains or clusters with > 3 cysts (Fig. 2). Chains of cysts were observed in 6 eyes (21%), clusters in 8 eyes (28%), and 5 eyes had both arrangement. The mean depth of cysts was 201 ± 126 μm (range 35 to 429). The depth of cysts was < 250 μm in 21 eyes (72%) and ≥ 250 μm in 8 eyes (28%).

Fifteen patients (52%) had therapeutic penetrating keratoplasty 63 ± 45 days (range 15 to 150) after the diagnosis. In univariate analysis, the factors associated with the worse outcome were a late-disease stage at presentation (OR 10.5, 95% CI 1.07–103.51), a deeper location of cysts (≥ 250 μm) (OR 11.38, 95% CI 1.17–110.42) and the presence of chains or clusters of cysts observed with IVCM (OR 14.86, 95% CI 1.53–144.22). No relationship was found between the outcome and demographic data, use of steroids, interval between onset of symptoms and diagnosis (Table 1). In multivariate logistic regression, only the presence of chains or clusters of cysts observed with IVCM remained associated with the worse outcome (OR 14.86, 95% CI 1.53–144.22). In univariate analysis, the deeper location of cysts was associated with the presence of chains or clusters of cysts (OR 19.00, 95% CI 2.54–141.93).

In one patient with chains and clusters of cysts observed with IVCM, Acanthamoeba cultures were examined under
In vivo confocal microscopy and Acanthamoeba keratitis

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Relationship between clinical findings, in vivo confocal microscopy features and outcome in 29 Acanthamoeba keratitis cases.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not requiring therapeutic penetrating keratoplasty</td>
</tr>
<tr>
<td></td>
<td>$n_{\text{total}} = 14$</td>
</tr>
<tr>
<td><strong>Delay between onset of symptoms and diagnosis</strong></td>
<td></td>
</tr>
<tr>
<td>$&gt; 1\text{month}$</td>
<td>7</td>
</tr>
<tr>
<td>$\leq 1\text{month}$</td>
<td>7</td>
</tr>
<tr>
<td><strong>Previous steroid use</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4</td>
</tr>
<tr>
<td>No</td>
<td>10</td>
</tr>
<tr>
<td><strong>Disease stage</strong></td>
<td></td>
</tr>
<tr>
<td>Late-disease stage (deep stromal keratitis or ring infiltrate)</td>
<td>8</td>
</tr>
<tr>
<td>Early-disease stages (epithelial or anterior stromal keratitis)</td>
<td>6</td>
</tr>
<tr>
<td><strong>IVCM depth of cysts</strong></td>
<td></td>
</tr>
<tr>
<td>$\geq 250\mu\text{m}$</td>
<td>1</td>
</tr>
<tr>
<td>$&lt; 250\mu\text{m}$</td>
<td>13</td>
</tr>
<tr>
<td><strong>IVCM arrangement of cysts</strong></td>
<td></td>
</tr>
<tr>
<td>Chains or clusters of cysts ($n &gt; 4$)</td>
<td>1</td>
</tr>
<tr>
<td>Scattered cysts ($n \leq 3$)</td>
<td>13</td>
</tr>
</tbody>
</table>

CI: confidence interval; OR: odd-ratio; IVCM: in vivo confocal microscopy.
Discussion

Numerous publications have reported the characteristic signs and have confirmed the usefulness of IVCM for the diagnosis of AK [4, 11]. As described previously, confocal microscopy findings of *Acanthamoeba* cysts showed double-walled, round- or stellate-shaped high-contrast particles and bright ‘signet ring’ images [4, 9–14]. Moreover, for some cysts, we observed a hypo-reflective halo surrounding cysts that may correspond to tissue edema. This hypo-reflective ring might also be explained by the optically empty space between the inner endocyst and the outer ectocyst when the peripheral wall of the classic “double-walled” cyst was not discernible from surrounding hyper-reflective structures (Fig. 2, bottom second image). Shrinkage of cysts induced by anti-infective drugs previously received by patients or even a different stage of *Acanthamoeba* might also explain this particular feature.

Although numerous studies have described the morphological IVCM aspects of cysts in AK, few have analyzed the arrangement of these structures. Our results showed that *Acanthamoeba* cysts were organized in chains and/or clusters in one third of patients with AK. It was consistent with the recent observation of Rezaei Kanavi et al. who showed “single file arrangement” of cysts in AK cases [15]. However, to our knowledge, no study has reported clusters of cysts or the association of clusters and chains of cysts in patients with AK in vivo. Interestingly, in one patient with late-disease stage we observed similar in vivo and ex vivo *Acanthamoeba* cyst features. Clusters of *Acanthamoeba* cysts have been described ex vivo, in cultures from patients with AK, but no chains were observed [16, 17].
Figure 2. In vivo confocal microscopy (IVCM) images showing the shape and arrangement of *Acanthamoeba* cysts (400 × 400 μm). Shape of *Acanthamoeba* cysts: double-walled cysts (arrow, top first image), ‘signet ring’ cyst (arrow, top second image), ‘bright spot’ cysts (arrow, top third image), stellate-shaped cysts (arrows, top fourth image) and hyper-reflective cysts with a peripheral hypo-reflective halo (arrowbottom first image). Cyst surrounded by hyper-reflective stroma (arrow, bottom second image). Arrangement of *Acanthamoeba* cysts: scattered cysts (bottom third image), chains (arrow) and clusters (arrowheads) of *Acanthamoeba* cysts (bottom fourth image).

Figure 3. Ex vivo laser confocal microscopy (EVCM) images showing *Acanthamoeba* in culture samples (400 × 400 μm). *Acanthamoeba* from a patient with in vivo confocal microscopy (IVCM) images of chains and clusters of cysts was cultured on non-nutrient agar overlaid with *Escherichia coli*. After 2 (top left), 4 (top right), 8 (bottom left) and 12 days (bottom right), growth was detected from the observation of *Acanthamoeba* cysts under confocal microscopy. Arrowheads indicate clusters of the cysts. Arrows indicate chains of cysts.
The advanced stage of AK in our case might explain these differences. In that previous ex vivo study, Acanthamoeba were only obtained from patients with an early-disease stage [16]. On IVCM images, Acanthamoeba cysts are difficult to differentiate from cellular debris, inflammatory cells or keratocytes [4]. Thus, the observation of chains or clusters of hyper-reflective structures could be an interesting new feature to identify cysts with IVCM in suspected cases of AK.

The arrangement of Acanthamoeba cysts in clusters or chains was independently and strongly associated with a worse outcome of AK in multivariable analysis. Patients with the particular IVCM aspect had a 15-fold increased risk to undergo therapeutic penetrating keratoplasty. The arrangement of cysts in chains or clusters might be a form of Acanthamoeba biofilm with a stronger virulence, resistance to the effects of antimicrobial treatments and escaping from the immunological response as it was described for bacteria or fungi [18,19]. This particular distribution may also correspond to different species more likely to induce severe keratitis. The association between this distribution of cysts and their location in deeper corneal tissues also suggested a higher degree of pathogenicity.

There was no statistically significant association between the arrangement of cysts and the disease stage. However, all patients with chains or clusters of cysts had a late-disease stage at presentation. The classification of disease-stage at presentation, into only two different stages and accounting for different severity grades, could explain the absence of statistically significant results. In accordance with previous studies, patients with late-disease stage at presentation had also a poor outcome as compared to early-disease patients [20,21]. However, in multivariate analysis, our results showed that the clinical stage at presentation was not associated with a poor outcome. The clinical evaluation of the disease stage may not only represent the quantity of infective microorganisms within the cornea but also the host immune response [20]. Moreover, the high prevalence of a traumatic etiology in our study, also observed in Indian studies, can influence outcome of AK [22]. Other prognosis factors such as the delay between onset and diagnosis had also been described in the literature [23]. However, consistent with the observations of Tu et al, this factor was not associated with a poor outcome in our study [20]. The evaluation of prognostic factors might be influenced by the different definitions of outcome measures or by the dependence between factors. Similarly to previous studies we defined patients with the worse outcome as patients requiring therapeutic penetrating keratoplasty [20]. Therapeutic keratoplasty has been reported as having poor results in AK [12]. Consequently this treatment was reserved for the most severe and advanced cases of AK not responding to medical treatment.

AK remains a challenging disease both in terms of diagnosis and treatment. Numerous studies have demonstrated the usefulness of IVCM as an adjunctive tool for the diagnosis of AK. By showing that a particular arrangement of cysts represents a poor prognostic factor in AK, IVCM may also help directly clinicians to evaluate the severity of the disease at presentation.

Disclosure of interest

The authors declare that they have no conflict of interest concerning this article.

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Involved in design of study (X.Z., X.S.); conduct of study (X.Z., X.S.); collection, management, and analysis of data (X.Z., A.L., C.J., Z.W., Y.Z., S.D.); interpretation and preparation of data (X.Z., A.L., C.J., Z.W., Y.Z.); and review and approval of manuscript (A.L., X.Z., X.S.).

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References

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