Endoscopic ultrasound guided fine needle aspiration allows accurate diagnosis of mycobacterial disease in HIV-positive patients with abdominal lymphadenopathy

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Abstract

Background:

 Abdominal lymphadenopathy in HIV remains a challenge due to inaccessibility of lymph nodes and multitude of causes. The diagnostic yield of EUS FNA in HIV-infected patients with abdominal lymphadenopathy in the setting of high tuberculosis (TB) prevalence was assessed.

Methods:

Prospective cohort study was conducted in tertiary referral centres recruiting symptomatic HIV+ patients (N=31, mean age 38.5 years, mean CD4 count 124 cells/μl, WHO stage 3-4 with abdominal

lymphadenopathy. EUS was performed to assess lymph node characteristics and FNA aspirate subjected to cytological analysis, microbial culture and PCR.

Results:

EUS appearance of lymph nodes was highly variable. *Mycobacterial* infections were the most common cause of lymphadenopathy in this cohort. Of the 31 patients 21/31 67.7 % had mycobacterial infections; 17 (80.9 %) of these were tuberculosis. Cytology failed to identify 23.8% and culture 38.1% of cases. PCR identified 16/17 (94.1%) of these cases. EUS-FNA altered the management of more than half of the patients.

Conclusions:

Mycobacterial disease was the commonest cause of lymphadenopathy in HIV but a third of patients had reactive lymphadenopathy. By combining the appearance of EUS FNA and cytological aspirate we could develop a diagnostic algorithm with a high PPV and NPV to identify patients in whom further analysis with PCR would be useful. PCR was highly accurate in confirming mycobacterial disease and determining genotypic drug resistance.

Key words: HIV, Abdominal lymphadenopathy, Endoscopic ultrasound, TuberculosisBackground

Isolated abdominal lymphadenopathy presents a diagnostic challenge in HIV- infected patients where a dysfunctional immune system may lead to atypical clinical presentations [1,2]. In endemic areas, *Mycobacterium tuberculosis* (MTB) accounts for the majority of cases of lymphadenopathy but needs to be differentiated from infections by atypical mycobacteria, other bacterial species, fungi and parasites, conditions such as reactive lymphadenopathy and various malignancies [2,3,4]. Imaging studies alone fail to distinguish between different etiologies [5], with the consequence of inaccurate diagnoses and empirical treatment decisions [6]. It is thus important to confirm the etiology of lymphadenopathy in HIV-positive individuals before commencing specific therapy [7].

Various methods have been described to obtain tissue samples from enlarged abdominal lymph nodes that vary in complexity and invasiveness. In general, percutaneous, endoscopic and surgical techniques are available [2,8,9,10]. Although surgical lymph node biopsy has been considered the gold standard [11], its invasive nature and related morbidity preclude its general use. Percutaneous-guided biopsies are often not feasible if lesions are in close proximity to major vessels or where there are overlying organs.

Endoscopic ultrasound (EUS)- guided fine needle aspiration (FNA) is a minimally invasive procedure that can be performed under conscious sedation, enabling the sampling of different lymph node

stations in the mediastinum and abdomen in a single session. EUS-FNA has become an established procedure in obtaining tissue from suspicious gastrointestinal and mediastinal lesions and has a low complication rate [11,12,13].

We hypothesized that in HIV, due to an attenuated dysfunctional immune response, the typical cytological findings associated with mycobacterial disease such as granuloma formation and the presence of acid fast bacilli may be absent or altered. This study examined the diagnostic efficacy of EUS-FNA in a population of advanced HIV-infected patients and the safety of the procedure in this setting. After obtaining EUS FNA samples the accuracy of various downstream diagnostic modalities were compared and the impact that the results of these investigations had on treatment decisions was examined.

Materials and methods

Study population

A prospective cohort of symptomatic HIV-1–infected individuals with abdominal lymphadenopathy were recruited between September 2009 to February 2012 from the HIV Comprehensive Care Clinic and from the Infectious Diseases Units, at Steve Biko Academic and Pretoria East Hospitals, Gauteng, South Africa. These tertiary referral centres specialize in advanced HIV care and therapeutic endoscopy. All patients seen in follow-up with symptoms suggestive of mycobacterial infection or malignancy were screened by ultrasonography or computed tomography for the presence of abdominal lymph nodes.

Patients were included in the study if abdominal lymph nodes >1 cm were present and if a microbiological diagnosis could not be established through standard examination of sputum, fluid, blood or superficial lymph node fine needle aspiration. Patients who were symptomatic despite at least three months of empiric treatment were also included. The most common symptoms observed in such patients were fever, weight loss and night sweats.

A presumptive diagnosis, based on the clinical and radiological information, was formulated by the infectious disease specialist. The endoscopist and pathologist were blinded to this. By comparing the presumptive diagnosis to the final diagnosis the impact of EUS FNA on decision making was examined. Due to the previously described absence of a diagnostic gold standard in such patients, a

diagnostic composite was employed that included cytological analysis, culture and PCR. This was done as per the NICE guidelines [14]. This protocol also followed the principles of the Declaration of Helsinki and was approved by the University of Pretoria Ethics Committee. Written informed consent was obtained from each participant.

EUS-FNA technique

The procedure was performed under conscious sedation. Linear array echoscopic ultrasound (Pentax Hitachi 7500) and standard 22-gauge needles (Cook Endoscopy, Limerick, Ireland) were used. The location of lymph nodes (celiac, porta-hepatis, retroperitoneal, para-aortic, mediastinal), number of nodes, echogenicity (hypo-echoic, hyper-echoic, isodense), form (round, matted, elongated), the absence or presence of necrosis, as well as the elastographic appearance were prospectively recorded. FNA was performed by trans-esophageal, gastric or duodenal approach depending on the location of the lymph node groups (figure 1 A-D).

The aspirate was characterized as 'bloody' or 'yellow' and smears were prepared and fixed on glass slides for cytological analysis. Additional aspirates were placed in three sterile tubes containing 500 µL of physiological saline for culture, PCR and flow cytometry. Post-procedure, patients were observed until stable and were monitored telephonically after 24 hours and at day 7 for possible procedure-related complications. Patients were followed for 3 months following the procedure to correlate endoscopic ultrasound findings with clinical outcome.

Cytology

Fine needle aspiration material was subjected to Papanicolaou staining (to evaluate morphology), as well as Giemsa, Ziehl-Neelsen, periodic acid-Schiff and Warthin-Starry stains. The cytological specimens were interpreted by an expert pathologist blinded to the presumptive diagnosis and endoscopic findings.

PCR technique

DNA was extracted from the aspirate using QIAamp® DNA Mini kits (Qiagen, Hilden, Germany). Detection of Mycobacterial DNA was performed using the Roche LightCycler® *Mycobacterium* PCR kit (Roche Diagnostics, Mannheim, Germany) allowing amplification and differentiation of *Mycobacterium tuberculosis* (MTB) complex from *Mycobacterium avium* (MAC) and *Mycobacterium*

kansasii by means of a post-PCR melt analysis. Genotypic rifampicin and isoniazid susceptibility testing was performed on MTB positive samples (Genotype® MTBDR*plus* assay, Hain Lifesciences, Nehren, Germany). The MTBDR*plus* assay is a PCR-reverse line probe assay that detects mutations associated with rifampicin resistance within an 81-bp 'hot spot' region of the *rpoB* gene. In addition, it detects mutations in codon 315 of the *katG* gene and in the *inhA* promoter region that are associated with INH resistance. This assay has been shown to have a sensitivity of >95% and >90% for detecting rifampicin and INH resistance respectively in South African TB isolates, as compared to the gold standard of phenotypic drug susceptibility testing [15].

A broad range fungal PCR targeting the conserved internal transcribed spacer region 2 (ITS-2) of the fungal ribosomal RNA gene was used to analyze each sample for the presence of fungal DNA. This PCR incorporated primers flanking the fungal ITS-2 region as described by Zeng et al [16]. The real-time PCR was performed with a 50µl reaction mixture containing 10µl extracted DNA, 25µl SensiMix dT (containing reaction buffer, heat-activated Taq DNA polymerase, dNTPs, 3mM $MgCl_2$) [Quantace, London, United Kingdom], 1x Sybr Green (Quantace, London, United Kingdom), 25pmol of primers its3Sb (5' GTGAATCATCGARTCTTTGAACG-3'; positions 271-279) and its4Ab (5'-GTTGGTTTCTTTTCCTCCGCTTAT TGATATGC-3'; positions 710-741). Primer positions refer to *Candida albicans* sequences corresponding to Genbank accession numbers AF455524 and L28817 respectively. Amplification was performed on a RotorGene 6000 thermal cycler (Corbett Research, Sydney, Australia), with the following conditions: 1 cycle of 94°C for 10 minutes, 30 cycles of 95 °C for 15s, 50°C for 30s, and 72°C for 30s. The presence of specific amplified product was determined by means of a post-PCR melt analysis. DNA sequences were compared with sequences from GenBank, EMBL, and DJB database using the gapped BLASTN 2.0.5 program.

Culture

Mycobacteria were isolated from the aspirated samples using BacT/ALERT liquid culture media (bioMérieux, Marcy-l'Etoile, France). Identification of positive cultures and genotypic rifampicin and isoniazid susceptibility testing were then performed. Non-tuberculous mycobacteria from positive cultures were identified using the GenoType® Mycobacterium CM reverse line probe assay (Hain Lifesciences, Nehren, Germany). Fungi were isolated from the biopsy samples by inoculation onto Sabouraud dextrose agar and incubation at 25°C for up to 3 weeks. Identification of isolates was performed by standard phenotypic methods.

Flow cytometry

Fine-needle aspiration specimens were washed twice with RPMI 1640 tissue culture medium, centrifuged at 250 g for 10 minutes. Enumeration of the cells was performed using Flow-Count™ Fluorospheres (Beckman Coulter, Miami, FL, USA) and the cells were then resuspended in RPMI 1640 tissue culture medium. Immunophenotyping was performed on a Beckman Coulter Cytomics FC500 (Beckman Coulter, Miami, FL, USA). Monoclonal antibodies were used to identify aberrant B and T-cell populations.

Statistics

All data were entered into a Microsoft Excel 2010 spreadsheet and descriptive statistics calculated. The accuracy of the diagnostic methods was assessed using two approaches. Firstly, due to the high burden of tuberculosis (TB) observed in this study, the ability of the methods to identify TB, and secondly, their ability to identify mycobacterial infections of any kind. That is, the results of the presumptive diagnoses and the individual diagnostic methods were binary classified in terms of their identifying any mycobacteria or TB in particular. In the TB-only analysis, we controlled for those patients undergoing prior anti-tubercular treatment (ATT) or in whom a lymphoma was found to be the final diagnosis. Using Statistix 9 (Analytical software, Tallahassee, Florida, USA) comparisons were made by means of cross tabulation, two-tailed Fischer exact tests and the calculation of positive and negative predictive values, between the results of each of the methods versus the final diagnosis. A p $\leq$ 0.05 value was considered significant.

Results

EUS-FNA assessment was conducted on 31 HIV-1-infected patients with pathologically enlarged abdominal lymph nodes. All patients had advanced HIV disease. The characteristics and the findings for the patients studied are given in table 1, with more detailed individual patient findings given in table 3 in the additional information.

EUS detected enlarged lymph nodes in all patients. Para-aortic and porta-hepatis nodes were the most common locations found in 20 (64,5 %) and 17 (54,8 %) of patients respectively, while 8 (25,8 %) of patients had additional mediastinal nodes. The endoscopic ultrasound appearance of lymph nodes was highly variable including partial evidence of necrosis (inhomogenous with central hypo-echogenicity) (32%), hypoechoic (48%), iso and hyper-echoic appearances (20%) (figure 1 A-D). Extra-nodal lesions were also found in 7 (22.5 %) of patients including: an infected sub-diaphragmatic multi-loculated cyst (5,6cm x 2,3cm) that was drained, one peri-pancreatic cold abscess and

pericardial effusions. Only one pericardial effusion was considered clinically important and was aspirated under EUS guidance where PCR and culture confirmed MTB. The procedure was found to be safe in this patient population and no immediate or post-procedural (48 hours) complications were reported.

The tissue samples obtained by EUS-FNA enabled a diagnosis in all patients. MTB was diagnosed in 17/31 (54.8%) patients, MAC in 5/31 (16.1%) and mixed TB/MAC in 1/31 (3%) of patients. In all but two MTB PCR positive patients, there was sufficient target DNA to perform direct drug sensitivity genotyping, leading to two patients being diagnosed with drug resistant tuberculosis. In all cases the final diagnosis was based on a combination of at least 2 of the following: cytology, culture and PCR. In one case, flow cytometry was unable to characterize a lymphoma and a surgical excision biopsy was required for confirmation of the subtype.

The accuracy of the diagnostic methods relative to identifying TB is given in table 2a. In the total patient group cytology, culture and PCR were all independently significantly associated with a Final_TB diagnosis. When the ATT-naïve patient group was compared, only culture and PCR remained significantly associated with the Final_TB diagnosis. The accuracy of the diagnostic methods relative to identifying *any* mycobacterial infection is given in Table 2b. A PCR (+) for mycobacteria, cytology-with a finding of necrosis and a positive culture of mycobacteria were all independently significantly associated with a Final_diagnosis of Mycobacteria. A finding of acid fast bacilli (AFB) was not. When cytology, AFB and culturing were combined into a composite variable, the resulting variable's diagnostic accuracy increased to nearly that of PCRs' and was significantly associated with a Final_mycobateria diagnosis. Following EUS-FNA aspirate evaluations, the final diagnosis changed from the presumptive diagnosis in a total of 13/31 (41.9%) patients and made additional diagnoses in 4/31 (12.9%) patients. The use of EUS-FNA therefore resulted in a change in the management of 17/31 (54.8%) patients.

In general, the main findings of this study were: i. Neither the size nor the EUS appearance could differentiate reactive from pathological lymph nodes ii. The cytological analysis of the aspirate alone had a low diagnostic accuracy even in patients not on anti-tuberculous therapy. iii. PCR had a high diagnostic accuracy in mycobacterial infection allowing a rapid diagnosis and early initiation of therapy. However, PCR failed to identify TB in one case where culturing was successful. iv. EUS FNA should ideally be succeeded with a differential diagnosis scheme in such patients.

Following the various procedures all patients were placed on treatment according to the final diagnosis and followed up for 3 months by study protocol before returning to scheduled clinic visits. One patient, diagnosed with 4 opportunistic infections, including *Cytomegalovirus* (CMV), *MTB*

pericarditis, *Mycobacterium avium complex* (*MAC*) and *C. Glabrata*, died during the observational period despite the initiation of appropriate therapy. All other patients stabilized or improved on specific therapy during the observational period

Discussion

The safety and diagnostic accuracy of endoscopic ultrasound in the assessment of mass lesions and lymphadenopathy in immuno-competent individuals is well established. In HIV-infected individuals the aberrant immune response may affect EUS interpretation of lymph nodes and the diagnostic accuracy of the aspirate. Such patients are predisposed to a multitude of opportunistic infections as well as hematological and other malignancies, frequently with atypical clinical presentations [1,2]. Additionally, cytological findings characteristic of bacterial infection, such as granuloma formation or the presence of acid fast bacilli, may be absent or altered [6].

In general, two strategies can be followed when managing HIV/AIDS infected patients with sputum smear negative tuberculosis with abdominal lymphadenopathy. Firstly, patients may be placed on empirical therapy targeting the most likely etiology given the local prevalence of disease. In MTB endemic settings, empirical therapy with anti-tubercular drugs may be initiated. However, ATT may be associated with adverse drug reactions, drug interactions, hepatotoxicity and, an empiric diagnosis of TB may miss other pathology [6]. Indeed, a study from India using EUS FNA to assess abdominal lymphadenopathy in HIV-uninfected patients, confirmed that almost half of the patients in this high endemic region were found to have non-tubercular pathology [17]. Similarly, our study confirmed that 31% of HIV infected patients with enlarged nodes, even in a high endemic area such as South Africa, had reactive lymphadenopathy. This implies that empirical ATT, prescribed solely on the basis of the presence of enlarged lymph nodes, may be associated with considerable treatment-related risks and should consequently be avoided.

Secondly, a tissue sample may be obtained allowing treatment decisions to be based on a laboratory confirmed diagnosis. Multiple sampling modalities are available including percutaneous FNA and minimally invasive surgery; neither has been extensively investigated in an HIV-positive population. A study from Thailand evaluated the role of percutaneous ultrasound-guided FNA in the assessment of abdominal lymphadenopathy in HIV-positive patients, reporting a high technical success rate and diagnostic accuracy. However, only patients with large, superficial nodes were included [2]. In patients with overlying organs, or smaller and deeper nodes, percutaneous sampling becomes

challenging [8]. Sampling of multiple lymph node stations may, furthermore, require multiple entry sites, adding to complexity and overall procedure time. In this study we report that endoscopic ultrasound is safe and effective in evaluating abdominal lymphadenopathy and obtaining tissue samples in HIV infected patients.

Classic EUS findings that have been proposed to differentiate between pathological and reactive nodes, include size greater than 1 cm, a round shape, hypo-echoic texture and sharp margins [18]. These findings were common in our study and not useful in differentiating between etiologies. Recent studies of mediastinal MTB in HIV-negative patients have found that the majority had hypo-echoic nodes [19], and that the lymph node appearance alone could not reliably differentiate tuberculous nodes from nodes due to sarcoidosis [20]. In our study the EUS appearance of lymph nodes in HIV-infected patients was highly variable, including a hypo-echoic texture, particularly when nodes smaller than 2cm were observed. Nodes also demonstrated central breakdown, extensive necrosis and with a matted or hyper-echoic appearance.

In HIV-uninfected patients, FNA aspirates obtained by EUS are routinely analyzed by cytology. A large study from India assessing mediastinal lymph nodes by EUS FNA based on cytology only, found an overall diagnostic yield of 93% [21].   In our study, however, findings typically associated with mycobacterial disease such as granuloma formation, were often attenuated, most likely due to HIV-associated immunosuppression. Additionally, although highly specific for mycobacterial disease, microscopy in the form of Ziehl-Nielsen staining for acid-fast bacilli (AFB), cannot reliably differentiate between MTB and MAC nor identify drug resistant strains. Culture has historically been considered the gold standard in identifying and differentiating mycobacterium species. However, it may take up to six weeks and is therefore of little value in early patient management, particularly in immune-compromised patients prone to rapid progression and early mortality [6].Owing to the high sensitivity and specificity of PCR for mycobacterial detection, its ability to differentiate between species and identify drug resistant strains [7], it has become part of the standard work up of MTB in addition to culture and cytology [22].

In high TB burden, resource limited settings it is desirable to avoid expensive and potentially unnecessary diagnostic tests. By determining the positive and negative predictive values (PPV, NPV), of the various post EUS-FNA modalities we were able to define a diagnostic algorithm (figure 2). Our results demonstrated that the presence of a yellow aspirate in combination with caseous necrosis on cytology had a PPV 96% where as a bloody aspirate in the absence of caseous necrosis had a 77% NPV which can be used to determine in which patients additional PCR should be performed. PCR remains imperative to differentiate MDR-TB strains and MTB from MAC .

Our detailed analysis was performed on a relatively small sample size of patients with advanced HIV infection. We did not include a control group of HIV-negative, TB-positive patients as more than 90% of new mycobacterial infections are diagnosed in HIV-positive individuals in South Africa. Irrespective, our study provides a clear description of the value of EUS and the utilization of PCR in the management of patients with advanced HIV with abdominal lymphadenopathy.

In summary, in addition to the safety and efficacy of EUS-FNA, the post procedural modalities of culturing in combination with PCR were found to be invaluable in providing a final diagnosis and guiding therapy. If mycobacterial infection was confirmed by PCR, sequencing and genotypic drug susceptibility testing could also be performed, enabling early diagnosis and the prompt initiation of appropriate therapy.

Figure legends

Figure 1:
Endoscopic ultrasound images of lymph nodes in the abdomen and mediastinum in advanced HIV patients. The appearances of lymph nodes are highly variable. (A.) EUS image of typical retroperitoneal lymph nodes in HIV populations contrasting the more hypo-echoic adrenal gland to the less hypo-echoic lymph nodes; (B.) Smaller nodes <2cm typically appeared round and hypo-echoic and simulated malignant nodes. (C). Mediastinal lymph node groups matted together so that individual lymph nodes cannot be distinguished were seen in a third of patients (D.) Presence of large peri-pancreatic in a patient negative by PCR and culture for mycobacterial disease. FACS analysis showed normal T- and B-cell populations. (E.) Multiple pathologies were detected in a quarter of patients. Here a pericardial effusion is shown that was PCR and culture positive for *Mycobacterium tuberculosis. Mycobacterium avium* was detected in lymph node aspiration by PCR and culture

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