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Original Article

A novel method to identify and isolate proliferative inflammatory atrophy (PIA) clusters and to extract high-quality PIA RNA

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Abstract: Epidemiological and histopathological studies have indicated that proliferative inflammatory atrophy (PIA) of the prostate is closely associated with the onset and development of prostate cancer (PCa). However, accurate isolation of PIA still remains a difficult matter, as well as high-quality RNA extraction from isolated PIA. These issues generated a lack of molecular evidence to support the mechanistic explanation proposed for the progression of PIA to PCa. Therefore, the isolation of PIA and the extraction of high-quality RNA from isolated PIA are of great importance to further demonstrate the correlation between PIA and the development of PCa at a molecular level. In this study, clinical samples from radical prostatectomy were stored in liquid nitrogen, PIA was identified by H&E staining of cryosections, PIA clusters were isolated by manual microdissection, total RNA was extracted from the PIA clusters by Trizol, and RNA quality was determined using the Agilent 2100 Bioanalyzer. Our results showed that PIA might be isolated by manual microdissection of cryosections stored in liquid nitrogen from clinical radical prostatectomy and used for extracting high-quality RNA (RIN > 7.5) by Trizol. Therefore, the present study established a valid method to discover molecular evidence in support of the correlation between PIA and the development of PCa.

Keywords: Proliferative inflammatory atrophy (PIA), prostate cancer (PCa), cryosection

Introduction

Prostate cancer (PCa) is one of the most common tumors of the urinary tract. According to the latest statistics issued by the National Institute of Health (NIH), the morbidity and mortality rates of prostate cancer in the US in 2013 are 238,590 with an average of 29,720 per year [1]. Although there is no clear statistical evidence, morbidity and mortality rates of prostate cancer is also rising in China due to the improvements in living standards, diet and lifestyle adjustments, and advances in diagnostic technologies [2]. Many studies have found that chronic inflammation contribute to the development of different types of cancer such as esophageal cancer, bladder cancer, liver cancer, stomach cancer, and colorectal cancer, through a variety of mechanisms [3, 4]. However, the relationship between chronic prostatitis and PCa is still unclear, although many evidences indicate that chronic prostatitis, especially non-resolving prostatitis, may very likely lead to PCa.

Centesis and clinical histopathological assessments of PCa patients showed that PCa is often found in association with proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN) [3, 5-7]. Karakiewicz *et al.* found that the majority of PCa patients have a history of prostatitis [6, 8-12]. Studies on the relationship between PCa and prostatitis show that the abnormal expression of scavenger receptor 1 (MSR1), macrophage inhibitory cytokine-1 (MIC-1), interleukin-8 (IL-8), interleukin-10 (IL-10), vascular endothelial growth factor (VEGF), intercellular adhesion molecule (ICAM), dendritic receptors, NF-κB, cyclooxygenase 2, and other inflammatory signaling genes is closely associated with the onset and development of PCa [5, 13-15]. Therefore, a patho-

logical model of PIA to PCa progression (PIA-PCa model) has been proposed. However, the difficulty in isolating PIA or extract high-quality RNA from isolated PIA, is precluding additional studies to discover molecular evidence in support of the PIA-PCa model.

The isolation of PIA and the extraction of high-quality total RNA from isolated PIA are essential requirements for the molecular biology analysis of the mechanisms of the PIA-PCa model. A number of procedures such as RNA sequence analysis, cDNA library construction, *in vitro* translation, and blotting assays require enough purity and integrity of RNA. Although RNA extraction is already a mature technique, the successful extraction of high-quality RNA from isolated PIA is still difficult for most skilled researchers due to the relatively small amount of PIA and PCa cells and their close proximity to each other. To address this issue, in this study we combined cryosection with manual microdissection to isolate PIA, improving the experimental conditions at all stages of RNA extraction, and analyzing the relationship between tissue preparation and the quality of extracted RNA. Hence, our study enables to achieve a high quality of both lncRNA expression profiling and PIA mRNA content, establishing a valid method to discover molecular evidences in support of the PIA-PCa model.

Materials and methods

Clinical tissue specimens

The prostate cancer tissue samples were collected from The First Affiliated Hospital of Nanchang University in 2013. The samples were immediately extracted from the abdominal cavity soon after excision, and washed with saline three times. Next, the samples were cut into 3 × 3 cm squares and immediately frozen in liquid nitrogen after removing necrotic, connective, and adipose tissues. The collection and study of tissue samples from all patients were both approved by the Medical Research Ethics Committee of The First Affiliated Hospital of Nanchang University. All tissue samples that were included in this study meet the following two criteria: 1) they belong to patients that have never received chemotherapy and radiotherapy, and 2) they were all pathologically confirmed after surgical resection to be PCa associated with PIA.

Identification of PIA by H&E staining of cryosections

The tissue samples were embedded in OCT cryomold (Sakura Finetek, Torrance, CA, USA) and placed in Leica CM1900 cryostat (Leica, Germany) at -28°C for 2 h. OCT embedding medium was removed from the samples using RNase-free water treated surgical blade, the samples were cut into to 6 μm-thick slices, and immediately fixed in 95% ethanol for 30 s to 1 min. H&E staining procedure was applied, the sections were dehydrated by passing through increasing concentrations of ethanol (70%, 80%, 90%, and 100%), cleared with xylene, mounted on neutral resin, and their tissue structure was examined under a microscope. The above steps were repeated until PIA was found.

Isolation of PIA by manual microdissection, extraction of total RNA, and determination of RNA quality

Once PIA was found following the procedure in Section 2.2, the approximate area of the PIA cluster was outlined with a marker under the microscope. The comparison with the sliced sections allowed us to locate and remove the tissue in excess using a surgical blade treated with RNase-free water (the removed area was 0.2-0.5 cm into the boundary of the marked area). The remaining tissue was cut into of 6 μm-thick slices, H&E staining was performed, and the slices were examined under a microscope to identify PIA. The above steps were repeated until PIA was the only remaining tissue that could be seen under the microscope. A total of 30-60 6 μm-thick slices were cut out; the number of slices was depending by the size of the PIA cluster. The completeness of the PIA cluster was determined in the middle and last sections. The slices that were not undergoing H&E staining were placed directly into Trizol reagent for RNA extraction. Agilent 2001 Bioanalyzer was used to estimate the quality of the extracted RNA.

Results

Identification of PIA by H&E staining

According to the criteria established by the 2006 International Working Group Classification System for Focal Prostate Atrophy Lesions, both simple atrophy (SA) and postatrophic hyperplasia (PAH) are classified as PIA. Epi-

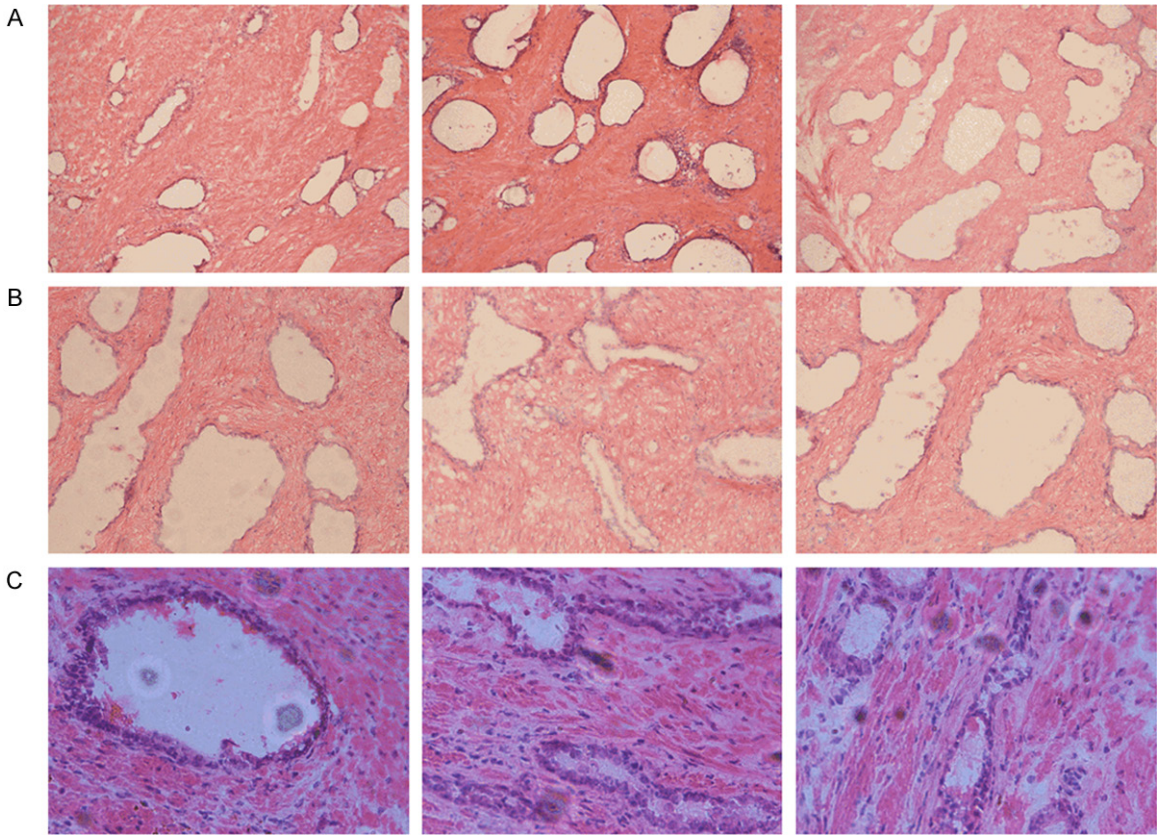

Figure 1. Microscope images of the PIA cluster, A: 100×, B: 200×, C: 400×.

thelial cells of atrophic lesions include the basal cell layer and the luminal cell layer. While the luminal cells in the atrophic lesions displayed a basophilic cytoplasm which was dark staining under microscope of the relatively number of. The luminal cells were flat with no clear cytoplasm. There were a few cytoplasms in the basal layer and many gaps (**Figure 1**).

Isolation of PIA by manual microdissection

The size of the selected tissue sections was 2 × 2.5 cm. The PIA clusters were identified under the microscope and isolated by manual microdissection. The size of the PIA clusters was approximately 0.2 × 0.15 cm. The PIA clusters were observed under the microscope in a full field-of-view to ensure that there were no normal and cancerous prostate tissues in the first, middle, and last slices (**Figure 2**).

RNA extraction (RIN > 7) by Trizol

The quality and concentration of the extracted RNA was evaluated using Agilent 2100 Bioanalyzer. The RIN values of the RNA from triplicate experiments subjected to H&E staining were 3.7, 4.3, and 2.6; the concentrations were 117, 116, and 164 ng/μl. The RIN values from samples that did not undergo H&E staining were 9.4, 9.3, and 8.6; the concentrations were 320, 371, and 66 ng/μl (**Figure 3**).

Discussion

Recent clinical and epidemiological studies strongly suggest that inflammation is closely associated with the onset, development, and metastasis of PCa [16]. PIA is a recently proposed concept, and is defined by De Marzo as the proliferation of epithelial cells on the basis of focal prostatic atrophy [17]. A number of laboratory and clinical data indicate that the onset and development of PCa follow a progression from PIA to PIN, followed by the malignant transformation into PCa, or even directly from PIA to PCa [18-20]. However, the existence of PIA in PCa is limited, and it is difficult to isolate PIA or to obtain high-quality total RNA and proteins from isolated PIA. Therefore, the pathological model of PIA to PCa progression could not be sufficiently validated.

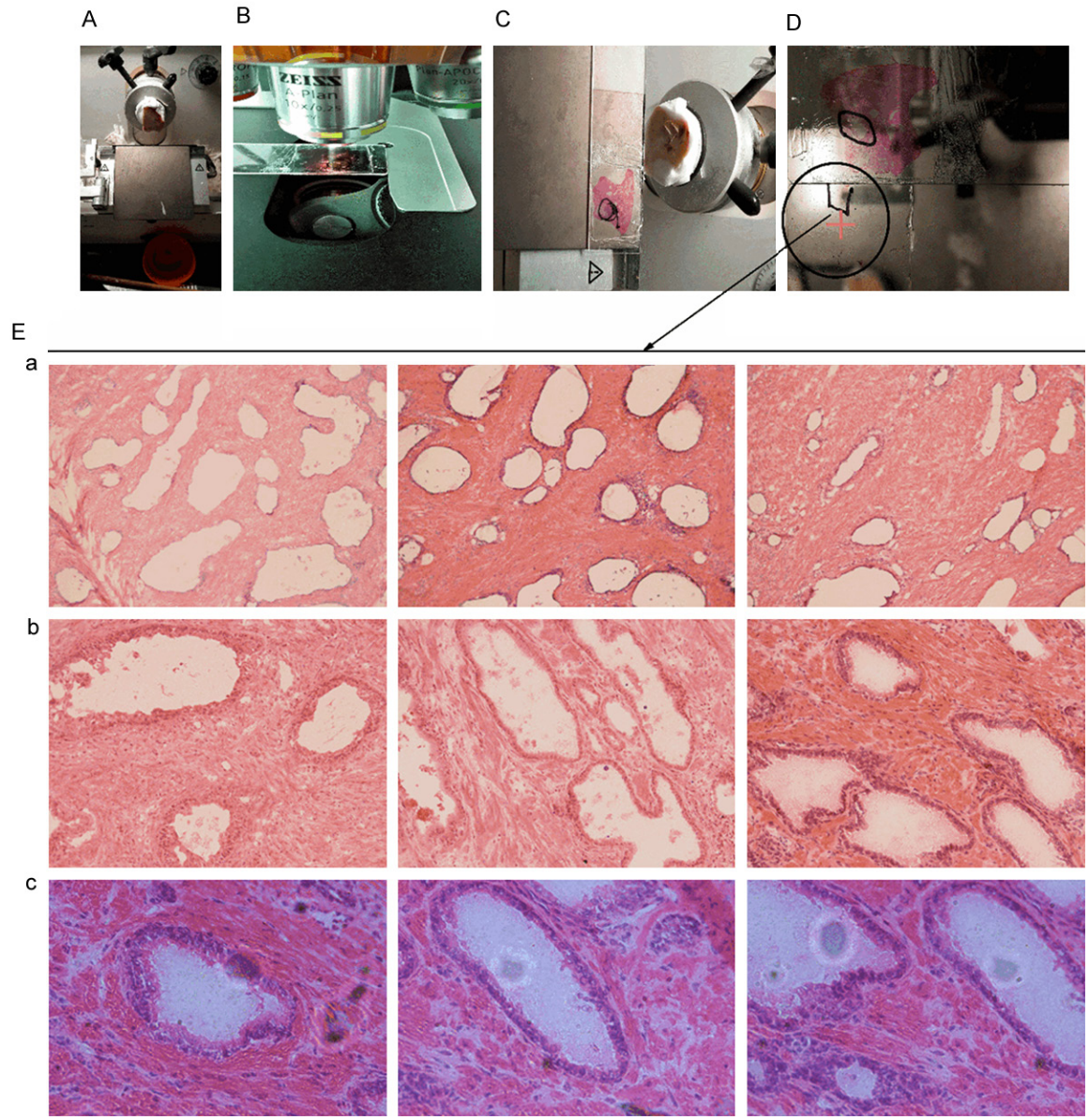

Figure 2. A schematic diagram showing the isolation of PIA by manual microdissection. A: Tissue section size before cutting, B: H&E staining of 6 μm-thick slices to locate PIA, C: The identification of PIA after H&E staining, D: Scheme of manual isolation of PIA clusters, E: Microscope images of PIA clusters after isolation. A: 100×, B: 200×, C: 400×.

Technological progresses in high-throughput microarrays and transcriptome sequencing have provided the basis for elucidating the mechanisms behind the onset and development of diseases [21]. However, high-throughput microarrays and transcriptome sequencing required a high quality of RNA, especially regarding the integrity and amount of RNA. For example, Agilent lncRNA plus mRNA Human Gene Expression Microarray V3.0 requires a total amount of RNA to be more than 1 μg with RIN greater than 7; TruSeq trace transcriptome and lncRNA sequencing requires a total amount of RNA more than 2 μg with RIN more than 7 [22-25]. Therefore, this restriction of high-throughput microarrays and transcriptome sequencing limits the high-throughput analysis of trace substances (such as PIA), thus preventing the analysis of pathological mechanisms through transcriptome data.

The aim of the present study was to discover a valid method for isolating PIA, for extracting high-quality RNA, and for the expression profil-

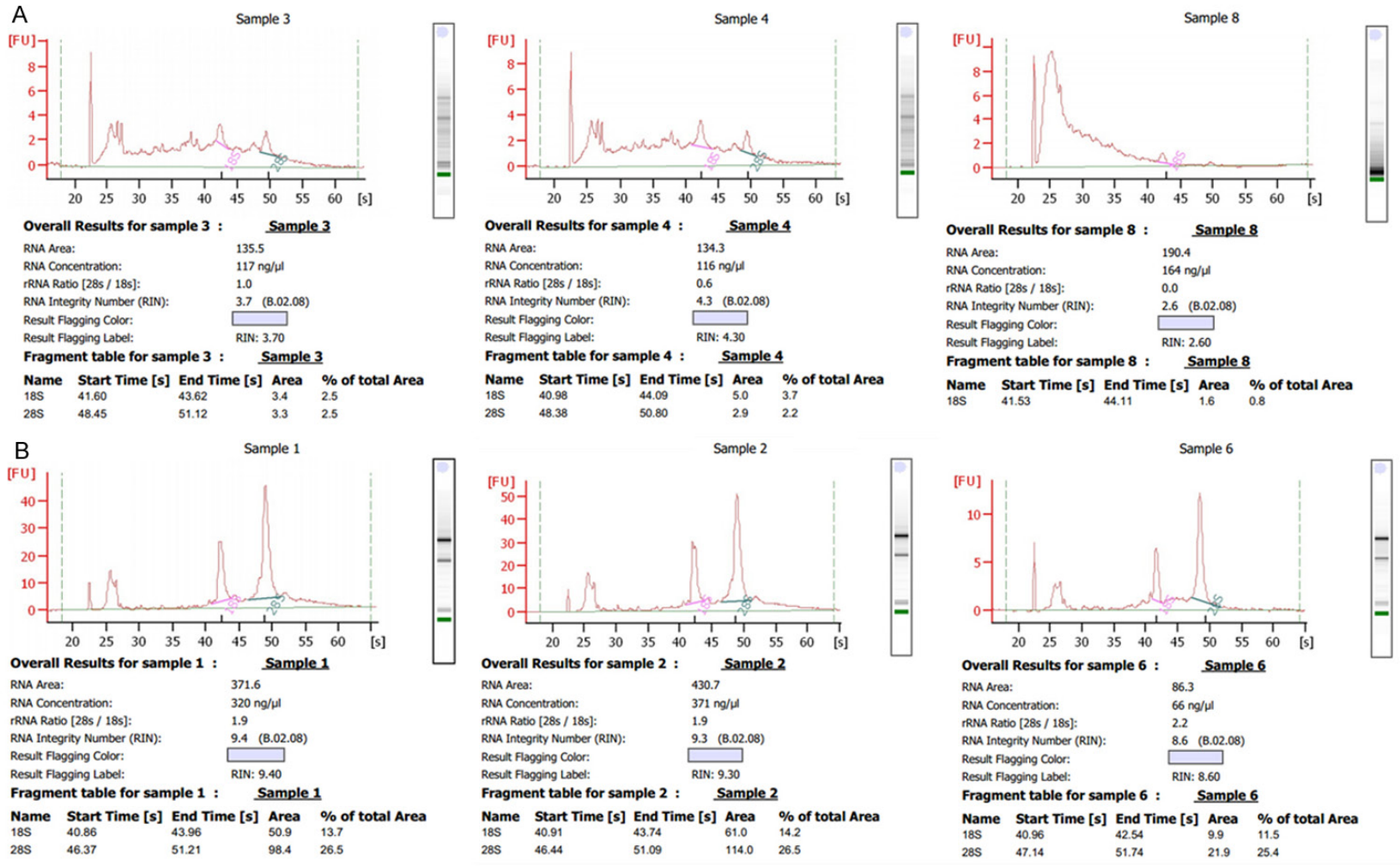

Figure 3. Evaluation of RNA quality and concentration using Agilent 2100 Bioanalyzer. A: RNA quality after H&E staining, B: RNA quality without H&E staining.

ing of mRNA and lncRNA content of PIA to validate PIA-PCa model from a molecular perspective. This study used manual microdissection to isolate tissue regions with PIA, which were define as PIA clusters. PIA clusters include cells within the atrophic lesion, such as basal cells, luminal cells, inflammatory cells, and interstitial cells between prostate glands. In order to ensure the purity of PIA clusters during their isolation by cryosection, the tissue specimens were sliced into 6 μm-thick slices and H&E staining was performed on the first, middle, and last slices to observe the purity of PIA clusters under a microscope. The analysis of the RNA quality and quantity revealed that RNA extracted from the PIA clusters using this method met the standards for high-throughput microarray and transcriptome sequencing. However, RNA extracted from H&E stained samples did not meet these standards. In conclusion, this study established a valid method for further transcriptome analyses to discover molecular evidence in support of the pathological model of PIA to PCa progression, as well as provided a method for the high-throughput characterization of other materials present in traces within tissues.

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Disclosure of conflict of interest

None.

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