Marine Pollution Bulletin 72 (2013) 94–98

Contents lists available at SciVerse ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Advanced diagnostics applied to fish liver tumours: Relating pathology to underlying molecular aetiology

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ARTICLE INFO

Keywords:
Laser microdissection
Frozen sections
Fish cancer
Tumourigenesis
Retinoblastoma gene

ABSTRACT

Liver tumours in flatfish have been diagnosed using histopathology for decades in order to monitor the impacts of marine pollution in coastal and estuarine environments. This traditional method has been coupled with molecular analyses of tumours in the liver of the dab, *Limanda limanda*, in order to elucidate underpinning molecular level aetiology of such disease. A laser capture microdissection technique for molecular investigation of cancer has been applied in fish. The present study provides optimized steps for environmental sample utilisation: a procedure for field sample collection and handling; a method allowing reliable identification of lesions on frozen tissue sections; and, downstream molecular analyses of tumourigenesis markers (*retinoblastoma* gene) in laser microdissected samples. This approach facilitates the use of wild flatfish as a model of environmentally-induced tumourigenesis, and has wide applicability to any disease pathology for which the underpinning molecular aetiology is required.

1. Introduction

Since the 1980s, liver pathologies of flatfish dab, *Limanda limanda*, English sole, *Parophrys vetulus*, and European Flounder (*Platichthys flesus*) have been used to monitor the effects of exposure to marine pollution (Malins et al., 1985; Vethaak and Ap Rheinallt, 1992; Stentiford et al., 2003; Lyons et al., 2004). Such lesions have been associated with exposure to anthropogenic contaminants such as polycyclic aromatic hydrocarbons (PAHs) (Malins et al., 1985; Vethaak and Ap Rheinallt, 1992). Dab possess both a similar histopathological tumour profile to humans (Stern and Zon, 2003) and homologs of human cancer genes such as *ras* and retinoblastoma (*Rb*), including mutational alterations of the *Rb* gene in tumour tissues (Du Corbier et al., 2005). In this respect, we have previously proposed that the dab tumour model could act as surrogate for cancer and the tumourigenesis process in human populations (Rotchell et al., 2009). Studies using this species also facilitate a better understanding of chemically-induced carcinogenesis in wild animals.

Histopathology of tumours and pre-tumours in dab liver are currently diagnosed via a quality assured process involving histological tissue sections generated from wax-embedded samples (Feist et al., 2004). Within the UK, such samples are collected and results are reported under the U.K. Clean Seas Environmental Monitoring Programme (CSEMP) (Stentiford et al., 2009). This diagnostic approach has recently been coupled with molecular analyses of tumour and surrounding non-tumour tissues (Small et al., 2010). In addition, gross lesions and apparently normal tissues have been resected from the dab liver for molecular investigations such as genetic alterations of cancer genes (Du Corbier et al., 2005; Rotchell et al., 2009), transcriptomic (Small et al., 2010), proteomic (Stentiford et al., 2009) and metabolomic studies (Stentiford et al., 2009; Southam et al., 2008). However challenges still remain, particularly in the ability to harvest pure populations of cells, to investigate the molecular profile of small lesions in the apparently non-tumour part of the liver, and to dissect small and early-stage tumours.

Microdissection and molecular analysis of pure populations of cells presents a challenge in that cellular heterogeneity within tissues samples may result in misleading findings (Cole et al., 1999; Sluka et al., 2008). Laser Capture-Microdissection (LCM) allows the ability to view and microdissect target cells, thereby providing a direct link between a specific histopathological lesion and the molecular profile of that lesion (Gillespie et al., 2001). Successful application of LCM is dependent on tissue morphology, capture success and maintenance of molecular integrity (Sluka et al., 2008). Compared to formalin-fixed paraffin embedded (FFPE) samples, cryogenically preserved samples are preferable for molecular analysis (particularly of RNAs) of LCM-derived samples (Gillespie et al., 2001; Sluka et al., 2008; Erickson et al., 2009). However, since the majority of diagnostic protocols in fish (and other hosts) are

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0025-326X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.marpolbul.2013.04.016

based upon use of FFPE-derived histological sections, an inherent difficulty in distinction of specific lesions types exist (Gillespie et al., 2001; Sluka et al., 2008; Vinas and Piferrer, 2008; Jorgensen et al., 2009). LCM has previously been applied to pathological studies in aquatic organisms including fish (Vinas and Piferrer, 2008; Jorgensen et al., 2009), cnidarians (Wiebring et al., 2010), and crustaceans (Small et al., 2008). While some LCM-based studies using frozen sections have been successful (Kitahashi et al., 2009), others have reported difficulty in the recognition of cells or lesions of interest within frozen sections mounted on membrane slides (Vinas and Piferrer, 2008; Jorgensen et al., 2009).

The present study aims at overcoming the challenges remaining in consistent application of LCM for molecular investigations of cancer in wild fish models. We have optimized field sample collection and handling at sea, the detection and classification of lesions on frozen tissue sections, and the downstream molecular analyses of carcinogenic markers in LCM-derived samples.

2. Materials and methods

2.1. Sample collection

L. limanda were captured at UK Clean Seas Environmental Monitoring (CSEMP) sites on the Dogger Bank, North Sea during July 2008 and 2009 using 30 min tows of a standard Granton trawl aboard the RV *Cefas Endeavour*. Upon landing, fish were immediately removed from the catch and placed into flow-through tanks containing aerated seawater. The sex, size (total length), and presence of external signs of disease were noted for each fish using methodology specified by the International Council for the Exploration of the Sea (Feist et al., 2004). A total of 72 fish were used in this study (of mean length 23.4 ± 2.4 cm; 51 individuals displayed either liver pre-tumours or tumours and 21 were normal). Following euthanasia, the body cavity was opened and the liver was assessed for the presence of macroscopic liver tumours according to the guidelines set out by Feist et al. (2004). In short, the liver was first examined in situ and a note taken of its size and colour. Next the presence of macroscopically visible nodules recorded. The maximum diameter of macroscopic nodules was recorded with notes on its general appearance, including texture, degree of surface vascularization and colour since the latter can occasionally give an indication of the histological nature of the nodule (Feist et al., 2004). For each fish, a standardised cross-section was obtained, placed into a pre-labelled histology cassette and fixed for 24 h in 10% neutral buffered formalin before transfer to 70% industrial methylated spirit. An additional tissue cross-section was also obtained from the site immediately adjacent to the formalin-fixed sample. The sample was embedded in Optimum Cutting Temperature (OCT) media (RA Lamb, U.S.A.) and frozen immediately in a cryobath containing iso-pentane and stored at −80 °C for subsequent laser-capture microscopy and molecular investigations. The sampling protocol allowed for a direct comparison of lesions in formalin fixed and cryopreserved samples of liver.

2.2. Sample processing

An overview of the sample processing regime is shown in Fig. 1. Formalin-fixed paraffin embedded (FFPE) samples of liver were prepared by vacuum infiltration processing by using standard protocols (Feist et al., 2004). Following embedding, sections were cut at 3–5 μm on a rotary microtome, and resulting tissue sections were mounted on glass slides before staining with haematoxylin and eosin (HE). Stained sections were analysed by light microscopy (Elipse E800, Nikon, U.K.) and the diagnosis of liver tumour type followed guidelines set out by Feist et al. (2004).

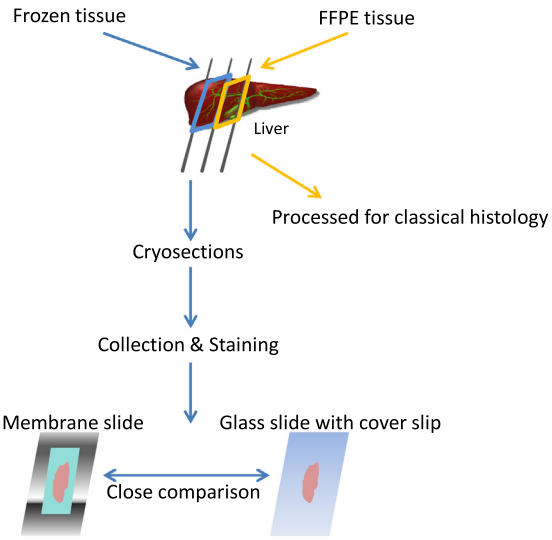

Fig. 1. Overview of the method used to diagnose lesions in frozen sections.

Appropriate frozen samples were selected according to the presence and identification of lesions in the corresponding FFPE sections. OCT-embedded liver samples were transferred to a cryostat adjusted to −20 °C. Samples were attached to a specimen disk with liquid OCT and allowed to freeze and harden within the cryostat chamber. For each liver sample, two frozen sections were cut and collected onto a clean glass slide. Sections were cut at 8 μm (a cutting thickness previously shown to give efficient yields of high quality RNA without an excessive increase in tissue opacity, or chance of dissecting unwanted cells). Sections were subsequently stained according to a protocol adapted from Huang et al. (2002). Glass slides were immediately placed into 70% ethanol for 2–4 min and then rinsed in DEPC water. Mayer's haematoxylin stain was applied to the slide surface for 2 min followed by a rinse in DEPC-treated water. The slides were then incubated in Scott's bluing solution (Leica Microsystems, U.K.) for 30 s followed by a rinse in DEPC water and a rinse in 70% ethanol. Eosin stain was applied for 45 s followed by dehydration in 95% ethanol for 30 s, in 100% ethanol for 1 min and two baths of xylene of 1 min each. Glass slides were mounted with DePex prior to microscopic evaluation. Frozen sections were screened to determine whether those lesions previously observed and classified within FFPE liver sections, were also present in the frozen sections. Specifically, the histopathological lesions of interest for the purposes of this study were: control (no abnormalities detected), vacuolated foci of cellular alteration (vFCA), eosinophilic FCA (eFCA), basophilic FCA (bFCA), hepatocellular adenoma (HCA) and hepatocellular carcinoma (HCC). Upon identification, digital images of histological lesions were obtained using the Lucia G Screen Measurement System (Nikon U.K. Ltd., Kingston-upon-Thames, U.K.). Two to five frozen tissue sections (depending on the size of the lesion) were collected onto membrane slides for LCM applications from fish that displayed no abnormalities and from lesions of interest in fish displaying tumours. Membrane slides were immediately placed in 70% ethanol on ice prior to staining as described above, followed by subsequent air drying. During this process, interspersed frozen sections were also obtained and collected onto glass slides (in between those collected for LCM) and stained as described above for further histological assessment. These additional sections allowed for the determination of the nature and localisation of the lesions (due to the reduced image quality when using LCM membrane slides) (Fig. 1). They also served as a record of the lesion type within a specific sample.