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The role of cancer genes in the development of haemic neoplasia in *Mytilus* sp.

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Abstract

Disseminated neoplasia has been reported in mussels (*Mytilus* spp) from numerous locations worldwide. This condition is progressive and fatal and the aetiology is unknown. In vertebrates, oncogenes such as *ras*, and tumour suppressor genes such as *p53*, play important roles in carcinogenesis. We have cloned *Mytilus* sp. homologues of the vertebrate *ras* and *p53* genes, both sequences conserved in regions of functional importance. Neoplastic blood samples derived from *M. trossulus* have been investigated for the presence of cancer gene mutations and changes in expression.

Keywords: Disseminated neoplasia, Oncogenes, Gene expression

Several types of malignant neoplasia occur in commercially important marine bivalves (Barber, 2004). One consists of a proliferation of abnormal circulating cells of

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unknown origin, referred to as “disseminated neoplasia”. Whilst disseminated neoplasia occurs in *Mytilus galloprovincialis* and *M. edulis* at low prevalences, in some areas it reaches epizootic prevalence in *M. trossulus* (up to 40% prevalence reported in Puget Sound, USA) causing serious regional economic damage to the aquaculture industry (Ciocan & Sunila, 2005). It appears that the bivalves *Crassostrea gigas* and *M. edulis* are resistant to disseminated neoplasia, yet the closely related species, *C. virginica* and *M. trossulus* are susceptible. A molecular aetiology for the observed differences in susceptibility to neoplasia in bivalves has been suggested (Van Beneden et al., 1999).

We have previously reported *M. edulis* cDNA sequences for the *ras* and *p53* cancer genes (Genbank No. AY679522 and AY705932)(Ciocan & Rotchell, 2005). Both display a high identity with vertebrate counterparts. Importantly, the mutational hot spots (at codons 12, 13, 59, 61 of *ras* and codons 245, 248, 249, 273 of *p53*) are conserved (Ciocan & Rotchell, 2005). Here, we report the isolation of cancer genes in *M. trossulus* and their involvement in haemic neoplasia.

M. trossulus were obtained from a mussel farm in Washington State, USA in April 2004. Haemolymph was withdrawn from the posterior adductor muscle, placed on a microscope slide coated with 0.1% poly-L-Lysine and incubated at RT for 10 min. The liquid was drained and the slide placed in methanol, then stained using a Feulgen picromethyl blue procedure. The remaining hemolymph was centrifuged (5 min, 300 g), the supernatant removed and pellets stored at -80°C . The hemolymph preparations were examined for the presence of neoplasms by viewing under a light microscope at 400x magnification.

All of the tumour samples that were analysed displayed advanced neoplasms in which neoplastic cells comprised greater than 95% of the cells present. Two forms of

neoplasms were observed (Moore et al., 1991); some were tetraploid while others appeared pentaploid (Fig. 1).

Total RNA was extracted from 9 normal and 9 tumour samples and cDNAs synthesised. PCR primers were: RAS forward, 5'ATGACGGAATACAAGCT3', reverse, 5'TCC TTCTCCCGTTCTCAT3', yielding a product of 231 bp; and P53RACE, 5'ATGAACCGSMGGCCCATYCTCACCATC3', yielding a 475 bp product. 18S rRNA expression was used as an internal calibration with primers: forward, 5'GTGCTCTTGACTGAGTGTCTCG3'; reverse, 5'CGAGGTCCTATTCCATTATTCC3', yielding a product of 172 bp. Amplification conditions were 35 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, with a final 2 min at 72°C. RACE conditions were: 5 cycles at 94°C for 5 s, 72°C for 3 min; 5 cycles of 94°C for 5 s, 70°C for 20 s, 72°C for 3 min; 25 cycles at 94°C for 5 s, 68°C for 10 s, 72°C for 3 min.

PCR products were also analysed on single strand conformation polymorphism (SSCP) gels to detect mutations. PCR products displaying extra bands were sequenced directly to characterize specific DNA changes.

The partial *M. trossulus ras* sequence isolated (Genbank No. DQ081184) showed high similarity to the *M. edulis* sequence (GenBank No. AY679522) differing at only 6 nucleotides, and without causing any change in the corresponding amino acid sequence. The expression of *ras* was induced in neoplastic samples compared with normal samples (Fig. 2). SSCP analysis also indicated extra bands and alterations in the *ras* gene in *M. trossulus* neoplastic samples. Direct sequencing confirmed *ras* gene polymorphic variation: two neoplastic samples displayed silent mutations at codons 13-15, 18-19, 21 24-25; one sample displayed a silent mutation at codon 70 alone; two further samples displayed silent mutations at codons 18-19 and 24. Most variation was

confined to the first exon and, in two cases, coincided with the mutational hotspot, codon 13. The normal samples displayed no *ras* gene polymorphic variation.

In contrast to *ras*, *P53* gene expression did not vary in neoplastic cells compared with normal cells. No mutations were detected in preliminary SSCP screening, though the fragment amplified excluded some traditional hotspots of mutational inactivation.

In summary, *M. trossulus ras* gene over-expression, though not mutational activation, may have contributed to neoplastic development. The significance of *ras* gene polymorphic variation in *M. trossulus* and the role of *p53* (as well as other tumour suppressor genes), in combination with *ras* gene expression, has yet to be elucidated.

Acknowledgements

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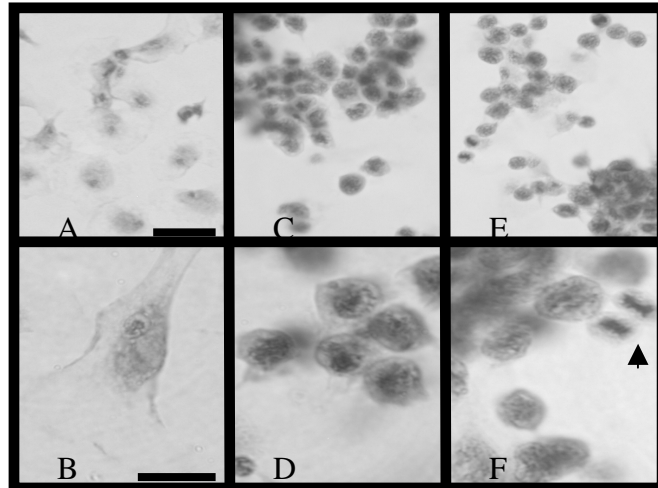


Fig. 1. Feulgen picro-methyl blue stained haemocyte preparations of normal and neoplastic *M. trossulus* samples. Scale bar in A,C and E is 20 μm ; in B,D and F is 10 μm . A,B: Normal haemocytes; C,D: Neoplastic cells with a small number of normal haemocytes; E,F: Neoplastic cells undergoing mitosis (arrow).

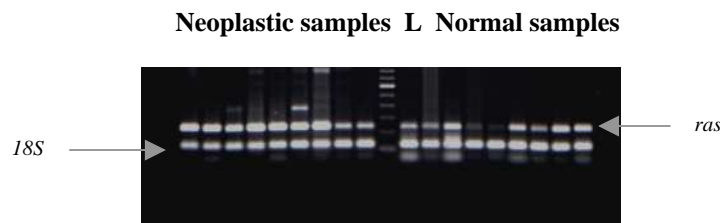


Fig. 2. PCR amplification of *ras* and *18S rRNA* genes from neoplastic and normal *M. trossulus* samples. L: molecular ladder.