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Gene expression profiling in melanised sites of Atlantic salmon fillets

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ABSTRACT

Black spots, which deteriorate quality of Atlantic salmon fillets represent a significant problem for commercial aquaculture. These areas are characterized with accumulation of melanomacrophages, occasional formation of granulomas and substitution of skeletal muscle with connective tissue. A number of possible causative agents have been suggested including vaccination and infection with piscine reovirus (PRV). We report transcriptome profiling of melanised foci with oligonucleotide DNA microarrays. Analyses revealed a multitude of differentially expressed genes associated with melanogenesis, metabolic changes and formation of scar. The immune profile was characterized with inflammation, preferential activation of classical complement pathway, MHCII and helper T cells combined with strong B cells responses and massive induction of immunoglobulins; innate antiviral responses were relatively weak in sharp contrast to PRV-caused heart and skeletal muscle inflammation and other viral infections. A panel of immune genes with specific activation in dark spots was found, most up-regulated were CD209-like lectin (44-fold) and prostaglandin reductase (11-fold). Further, RNA sequencing was performed on the same material to search for the presence of putative pathogens. Transcripts of prokaryotic rRNA with exclusive or preferential location in black spots were found. Results suggest mild chronic inflammation initiated with trauma, bacterial or viral infection followed by sustained immune responses to opportunistic microorganisms as a realistic scenario of dark spots formation.

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1. Introduction

Salmon fillets with deviating appearance are discarded by the consumers and processing industry [1]. At present superficial hyper pigmented areas of salmon fillets or “black spots” (BS) represent a major problem for the commercial aquaculture of Atlantic salmon. The prevalence of BS on fillets of Norwegian sea reared salmon has increased from 7% in 2003 to 20% in 2015 [2]. Costs associated with extra labor due to manual removal of dark stained muscle and quality degradation exceed 100 million Euros a year [3]. BS, which are most commonly located cranio-ventrally below the horizontal septum, develop at sites of melanomacrophage centres (MMCs) – groupings of pigment containing cells. In salmonids, MMCs have a high proportion of dark pigments, including melanins [4], and the interaction between the pigmentary and immune systems has been associated with abnormal melanisation of chronically, granulomatous inflamed skeletal muscle [5–7]. BS in salmon have been characterized with the presence of melanomacrophages, T-cells and MHC class II + cells dominating the leukocyte infiltrates in the inflamed tissues [6,8]. Degenerative processes can occur simultaneously with regeneration accompanied by enzymatic *de novo* production of melanin [6,9].

Several attempts have been undertaken to investigate aetiology of BS in salmon muscle. Vaccination [5] and interaction between vaccination and smoltification [10] have been discussed as possible causes. Larsen et al. [6] did not detect pathogens in BS and supposed that ectopic melanin deposition in salmon skeletal muscle could be due to non-infectious, foreign-body type of granulomatous inflammation. Lerfall et al. [11] found hyperpigmented areas of larger size in skeletal muscle of salmon infected by salmon alphavirus 3 (SAV3), although a high variation was observed within and between salmon groups diagnosed with pancreas disease (PD) and groups with no records of PD diagnosis. Recently, Bjørgen et al. [8] concluded that acute inflammation of salmon fillets with red colouration that can progress to a melanised pathotype was associated with the presence of piscine reovirus (PRV); infection with PRV was

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suggested as a premise for the development of focal melanised changes. This hypotheses stimulated active discussions in aquaculture industry and research. However, despite association with BS, neither PRV nor SAV are sufficient for their development suggesting contribution from other pathogens or non-infectious agents. The aim of this work was to explore the molecular events related to BS on the cranio-ventral part of Atlantic salmon fillets. Transcriptome analyses were carried out with the Atlantic salmon oligonucleotide microarray [12] and deep RNA sequencing (RNA-seq). In particular, RNA-seq was performed to provide a means for searching for possible infectious agents in the hyper-pigmented areas. We present results that question association between BS and PRV or any other viral pathogen.

2. Material and methods

2.1. Ethics statement

Rearing and slaughtering were conducted at Nofima's Research station (Averøy, Norway) which is approved by the Norwegian Animal Research Authority (NARA). Stunning and harvesting of fish were performed in accordance with the Norwegian Animal Welfare act. Fish were treated as production fish up to the point of tissue sampling which was done only after fish were put to death.

2.2. Fish material and sampling

The fish examined were clinically healthy farmed Atlantic salmon with known pedigree, obtained from the breeding nucleus of SalmoBreed AS, Norway. The fish were routinely intraperitoneally injected with oil-adjuvanted vaccine before seawater transfer following standard procedures (Sommerset et al., 2005) in May 2012 at Nofima's land based research station. The fish were harvested in February 2013 by percussive stunning, bled to death in running seawater after cutting the gill arches and immediately gutted and filleted. The salmon subjected to analyses (average body weight 4.2 kg) were selected among fillets with dark stained spots on the cranio-ventral fillet part, below the horizontal septum. Dark stained skeletal white muscle and neighbouring apparently normal muscle (2–3 cm beside the dark pigmented muscle) were collected from twenty fish and stored in RNALater (Ambion). Among those, twelve and six individuals with minor to major pathology were selected for transcriptomic analyses and RNA sequencing, respectively.

2.3. RNA isolation

RNA was isolated using the PureLink RNA Mini Kit (Invitrogen Corporation, Carlsbad, CA). Tissue was homogenized in TRIzol in a Precellys 24 (Bertin technologies). RNA integrity was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies).

2.4. Microarray analyses

Gene expression profiling was performed with Nofima's Atlantic salmon oligonucleotide microarray SIQ-6 (GPL16555) fabricated by Agilent Technologies in the 15 K × 8 format, all reagents and equipment were purchased from the same source. Dual-label hybridizations were carried out. Individual samples (Cy3) were hybridized to a pool of all twelve nonpigmented samples used as a common reference (Cy5). RNA libelling and amplification were performed with Low Input Quick Amp Labelling Kits, Two-Colour and RNA Spike-In Kits, Two-colour using 200 ng of total RNA per reaction. For fragmentation of the labelled RNA, Gene Expression Hybridization Kits were used. Labelled RNA was hybridized for 17 h in an oven at 65 °C and rotation speed of 10 rounds per minute. Arrays were washed for one minute with Gene Expression Wash Buffer I at room temperature, and one minute with Gene Expression Wash Buffer II at 37 °C. Slides were scanned using GenePix Personal 4100A scanner (Molecular Devices, Sunnyvale, CA, USA) at 5 mm. The GenePix Pro software (version 6.1) was used for spot-grid alignment, feature extraction and assessment of spot quality. Subsequent data analyses were carried out with Nofima's bioinformatics package STARS [12]. Low quality spots were filtered by flags assigned with GenePix. Log_2-Expression Ratios (ER) were calculated and Lowess normalization was performed. The differentially expressed genes were selected at the cut-off values: 2-fold ER and $p < 0.01$ (one sample *t*-test). For comparison of gene expression profiles in BS and in cardiac muscle of fish with heart and skeletal muscle inflammation (HSMI) [13], the differentially expressed immune genes were grouped by the functional roles and the mean log_2-ER were calculated (all analyses were carried out with SIQ-6 oligonucleotide microarray).

2.5. RNA-seq and sequence analyses

Total RNA was first depleted from salmon rRNA using RiboMinus Eukaryote kit for RNA-seq (ThermoFisher Scientific, Waltham, MA, USA) as per manufacture's instructions. Transcriptome libraries were prepared by the Genotypic Technology's Genomics facility (Genotypic Technology Pvt. Ltd., Bangalore, India) using Illumina TruSeq RNA library preparation kit. Each sample was indexed with a separate barcode and pooled into a single library for sequencing. The libraries were sequenced on an Illumina NextSeq platform using 75 base pair, paired-end reads (PE). FastQC along with trimmomatic [14] were used for assessments and trimming low quality bases in order to retain only the highest quality nucleotides. Reads were mapped to the Atlantic salmon reference assembly (ICSASG_v2) using Bowtie2 (version 2. 2.5) [15] and TopHat (version 2.0.13) [16]. Cufflinks (version 2.2.1) [17] was used to guide transcript assembly and to obtain gene and transcript level expressions. Transcript abundances were quantified and genes showing minimum of 2-fold difference in their expression with an adjusted p value < 0.05 were defined as differentially expressed. All the reads that failed to align to the Atlantic salmon genome, were further compared against the NCBI nucleotide and NCBI rRNA databases using the blast tools (cut-off e-value 10^{-10}) [18] and bowtie2 respectively. The count data were then normalized and assessed for differential abundance patterns between the BS and the normal tissues using the R Bioconductor package, edgeR (version 3.10.5) [19].

3. Results

3.1. Microarrays

Microarray analyses revealed profound differences between BS and unchanged muscle: 1570 genes showed greater than 2-fold expression changes, of which 946 genes were up-regulated. Despite different levels of pigmentation intensity, the gene expression changes in all analysed samples were highly similar as witnessed by correlation between the individual and the mean expression profile (Pearson $r = 0.93 \pm 0.02$). A large fraction of differentially expressed genes (423 features) were specialized immune genes and major part of them (396 features) were up-regulated. Diverse aspects of innate and adaptive immunity were affected (Table 1). Since systemic reactions commonly involve most if not all functional groups and pathways of immunity, rather proportions between the components than absolute expression changes can be informative with respect to the character of the

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