Developmental and tissue-specific expression of NITRs

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Abstract Novel immune-type receptors (NITRs) are encoded by large multi-gene families and share structural and signaling similarities to mammalian natural killer receptors (NKRs). NITRs have been identified in multiple bony fish species, including zebrafish, and may be restricted to this large taxonomic group. Thirty-nine NITR genes that can be classified into 14 families are encoded on zebrafish chromosomes 7 and 14. Herein, we demonstrate the expression of multiple NITR genes in the zebrafish ovary and during embryogenesis. All 14 families of zebrafish NITRs are expressed in hematopoietic kidney, spleen and intestine as are immunoglobulin and T cell antigen receptors. Furthermore, all 14 families of NITRs are shown to be expressed in the lymphocyte lineage, but not in the myeloid lineage, consistent with the hypothesis that NITRs function as NKRs. Sequence analyses of NITR amplicons identify known alleles and reveal additional alleles within the \textit{nitr1}, \textit{nitr2}, \textit{nitr3}, and \textit{nitr5} families, reflecting the recent evolution of this gene family.

Keywords Innate immunity · Lymphocytes · Natural killer receptors

Novel immune-type receptor (NITR) genes have been identified in zebrafish and 14 other bony fish species (Yoder 2009; Ferraresso et al. 2009). Several lines of investigation suggest that their occurrence may be restricted to teleost species. The majority of NITR transcripts encode type I transmembrane proteins. All NITRs possess a single extracellular immunoglobulin (Ig) domain of the variable (V)-type and most possess a second Ig domain of the intermediate (I)-type. NITRs can be classified as: inhibitory, activating and functionally ambiguous based on peptide signaling motifs (Yoder 2009). Bony fish possess cytotoxic NK-like cells (TCR$\alpha^+\beta^-\gamma^+\text{IgM}^-$) as well as cytotoxic T cells (TCR$\alpha^-\beta^+\text{IgM}^-$) (Shen et al. 2002) and inhibitory and activating NITRs may play significant roles in the functional regulation of both of these lymphocyte populations (reviewed in Yoder 2009). A phylogenetic comparison of
NITR sequences from different fish species demonstrates that these genes are both recently and rapidly evolving (Yoder 2009). Although the fundamental role of NITRs is not known, their specificity for allogeneic determinants, a characteristic of many NK receptors, has been demonstrated (Cannon et al. 2008).

The expression levels of subsets of NITR genes have been described in various tissues in multiple bony fish species and NITR transcripts are detected predominantly in hematopoietic tissues (Strong et al. 1999; Hawke et al. 2001; Yoder et al. 2002; Piyaviriyakul et al. 2007; Evenhuis et al. 2007; Ferraresso et al. 2009). The majority of NITR transcripts are between 1.4 to 2.0 kB as detected by RNA blot analyses. NITR expression in spleen, kidney and intestine is more abundant than in muscle and liver (Strong et al. 1999; Hawke et al. 2001; Yoder et al. 2002; Evenhuis et al. 2007; Ferraresso et al. 2009). NITRs also are expressed in circulating leukocytes of trout and sea bass (Yoder et al. 2002; Ferraresso et al. 2009) as well as in gills of Japanese flounder and sea bass (Piyaviriyakul et al. 2007). RT-PCR analyses reveal that several different classes of NITRs are expressed at different levels in different tissues (Strong et al. 1999; Yoder et al. 2002; Piyaviriyakul et al. 2007; Evenhuis et al. 2007). Furthermore, expression of NITR transcripts has been detected by RNA blot and RT-PCR analyses in various clonal NK-like, T, B, and macrophage cell lines of channel catfish (Hawke et al. 2001; Evenhuis et al. 2007). Certain NITRs in catfish appear to be expressed nearly ubiquitously in the various hematopoietic cell lines whereas expression of other catfish NITRs is more restricted (Evenhuis et al. 2007).

Thirty-six zebrafish NITR genes representing 12 families of NITRs have been identified from the zebrafish genome in a single gene cluster on chromosome 7 (Yoder et al. 2004). Three additional NITR genes, defining two additional families, are encoded in a gene cluster on zebrafish chromosome 14 (Yoder et al. 2008). Neither the tissue

![Fig. 1](image-url) Overview of oligonucleotide primer design for reverse-transcriptase polymerase chain reaction (RT-PCR). Primer sets are listed on the left. Genes targeted by each primer set and the overall genomic organization of these genes are listed to the right of each primer set. Families of NITRs are defined by number (e.g., nitr1). Individual genes within a family are identified with the addition of a lowercase letter (e.g., nitr1a). Alternatively spliced isoforms are identified with uppercase letters L, S, and SS for long, short, and super-short, respectively (e.g., nitr9L). Colored rectangles represent exons and black arrows approximate the relative location of each primer. The peptide domains associated with each exon are shown at the bottom as gray rectangles (L peptide leader sequence, V variable domain, I intermediate domain, TM transmembrane domain, Cyt1 and Cyt2 cytoplasmic regions). Primer sequences are listed in Table 1.
distribution nor expression of the different NITR families has been examined in a comprehensive manner. We describe herein the expression patterns of 14 different NITR families in zebrafish embryos, tissues and leukocytes. In order to detect all possible NITR transcripts, including described polymorphisms and alleles, a panel of primers was developed for detecting NITR transcripts by RT-PCR. For example, three degenerate primer pairs were used to

| Primer sets | Forward primer | Reverse primer | Annealing Temp (°C) | Extension time (s) | No. of cycles (Fig. 2) | No. of cycles (Fig. 3) | Amplicon length (bp) |
|-------------|----------------|----------------|---------------------|-------------------|-----------------------|-----------------------|---------------------|
| nit1a/b/c/f/k | AAGACAAGGCTYYAGACTCTGC GGGTGGTCTCGGAAGCTTGGA | AAGACAAGGCTYYAGACTCTGC GGGTGGTCTCGGAAGCTTGGA | 65 | 30 | 40 | 40 | 345 |
| nit1d/g/h/i | TCAGACTCTGCAACATATTACCTGTG ACACGACAGATGAACACACTGA | TCAGACTCTGCAACATATTACCTGTG ACACGACAGATGAACACACTGA | 65 | 30 | 40 | 40 | 367/370 |
| nit1j/l/m/n/o | GGTATATTTATATCACCATTATTAAA TAAATGCCCGWATCAGACGCASACTGA | GGTATATTTATATCACCATTATTAAA TAAATGCCCGWATCAGACGCASACTGA | 60 | 30 | 40 | 40 | 389/392/395 |
| nit2a/b/d/e | GATGWGKYAGTTTTAATCTGAGACATC GCAGGCTGTAGATGCATTTGTGTGACAG | GATGWGKYAGTTTTAATCTGAGACATC GCAGGCTGTAGATGCATTTGTGTGACAG | 65 | 30 | 40 | 40 | 346 |
| nit3a/c | GARGAWTTTGGCAAMYTAYTATTG GTAAGCTTTATGTTTGGCAAMYTAYTATTG | GTAAGCTTTATGTTTGGCAAMYTAYTATTG GTAAGCTTTATGTTTGGCAAMYTAYTATTG | 55 | 30 | 40 | 40 | 378 |
| nit4 ORFb | ATGATCAACATCATCATCAGTATCATC TCACTCGTCGACGCTTGCTGCTGTCG | ATGATCAACATCATCATCAGTATCATC TCACTCGTCGACGCTTGCTGCTGTCG | 65 | 30 | 40 | 40 | 957/1014 |
| nit5 | GAAGAGCCACGCTTCACTTATAGTCGC ATGATCAACATCATCATCAGTATCATC TCACTCGTCGACGCTTGCTGCTGTCG | GAAGAGCCACGCTTCACTTATAGTCGC ATGATCAACATCATCATCAGTATCATC TCACTCGTCGACGCTTGCTGCTGTCG | 70 | 30 | 35 | 35 | 354 |
| ntr6a/b/c | TTGGKTYATTMTGTGTTGTKGCC TKATGTTGAGTGAAAGAGATCGCTT | TTGGKTYATTMTGTGTTGTKGCC TKATGTTGAGTGAAAGAGATCGCTT | 58 | 30 | 40 | 40 | 284 |
| nitr7a/b | TCTGGAACCTACAGCCGCTGATGCTC CATCCAGCAGCTGATATCCTCGATGTCG | TCTGGAACCTACAGCCGCTGATGCTC CATCCAGCAGCTGATATCCTCGATGTCG | 70 | 30 | 40 | 40 | 262/301/304 |
| nitr8 | CAACATGACAGCAGYTTAATATCCTCTC TTCTATTATCTCTCTCAGGTAATCTCAC | CAACATGACAGCAGYTTAATATCCTCTC TTCTATTATCTCTCTCAGGTAATCTCAC | 65 | 30 | 35 | 35 | 486 |
| nitr9 ORFb | ATGATCAACATCATCATCAGTATCATC TCACTCGTCGACGCTTGCTGCTGTCG | ATGATCAACATCATCATCAGTATCATC TCACTCGTCGACGCTTGCTGCTGTCG | 65 | 30 | 40 | 40 | 606/858/951 |
| nitr10a/b | GCAGCTTTATGCTCTTGTGCCACCAAGAAACCAGGTTAATCAT | GCAGCTTTATGCTCTTGTGCCACCAAGAAACCAGGTTAATCAT | 65 | 30 | 40 | 40 | 323 |
| nitr11a/b | GYGTKTAKATAAYTTCTAGCGCTTGTGATG ATTTCACAGGAMCACAAGCACAAGCGAAG | GYGTKTAKATAAYTTCTAGCGCTTGTGATG ATTTCACAGGAMCACAAGCACAAGCGAAG | 65 | 30 | 35 | 40 | 348/351 |
| nitr12 | GCACACCTGGAGAAGCTCACAACGAGGCTGATG | GCACACCTGGAGAAGCTCACAACGAGGCTGATG | 65 | 30 | 40 | 40 | 502 |
| nitr13 | GAGATTCTCTCATGCTCTATCGAGG CATCAGCGAATATGTCGTTG | GAGATTCTCTCATGCTCTATCGAGG CATCAGCGAATATGTCGTTG | 70 | 30 | 40 | 50 | 328 |
| nitr14a/b | ATGATTCCTCGGACATTGTTACTG ATGATTCCTCGGACATTGTTACTG | ATGATTCCTCGGACATTGTTACTG ATGATTCCTCGGACATTGTTACTG | 60 | 30 | 40 | 40 | 326 |
| LC1 | GAAGATGCTTCGGAGATTAATACTGT ACTCTGAAACTCCTGTTGTTT | GAAGATGCTTCGGAGATTAATACTGT ACTCTGAAACTCCTGTTGTTT | 65 | 30 | 40 | N.A. | ~433 |
| LC3 | GAAGATGCTTCGGAGATTAATACTGT ACACACAGTACGACTGTTTG | GAAGATGCTTCGGAGATTAATACTGT ACACACAGTACGACTGTTTG | 65 | 30 | 40 | N.A. | ~405 |
| TCRα | GCAATNTAYATGTYGC ATGATCCATGACAAGAG | GCAATNTAYATGTYGC ATGATCCATGACAAGAG | 70 | 30 | 40 | 50 | 328 |
| mpx | CCAGAACACGGTAGGAGACGACAGCA CAGTCTACACAGGACGAGCTG | CCAGAACACGGTAGGAGACGACAGCA CAGTCTACACAGGACGAGCTG | 70 | 30 | 40 | 50 | 639 |
| β-actin | GGTATGGAACCTCTGCTGTCTCCTTCTC | GGTATGGAACCTCTGCTGTCTCCTTCTC | 65 | 30 | 25 | 25 | 301 |

*a Touch-down PCR during which the annealing temperature is lowered from 65°C to 55°C by 0.5°C per cycle for 20 cycles and then an additional 20, 25, or 30 cycles are completed with a 55°C annealing temperature

*b Sequences corresponding to translational start and stop codons are underlined
detect the 14 member genes of the \textit{nitr}1 family (Fig. 1 and Table 1). As observed previously in other bony fish species, all zebrafish NITRs are expressed in the kidney, spleen and intestine; a few NITR transcripts are expressed at lower levels in the liver (Fig. 2). Notably, transcripts of the \textit{nitr}1, \textit{nitr}3, \textit{nitr}6, \textit{nitr}7, \textit{nitr}9, \textit{nitr}10, \textit{nitr}12, and \textit{nitr}14 families were detected in the ovary. However, only maternal transcripts of \textit{nitr}3, \textit{nitr}7, and \textit{nitr}9 were detected in the 1-cell embryo (0-h post-fertilization) and only \textit{nitr}3 was expressed throughout embryogenesis. Efforts to further characterize \textit{nitr}3 expression during embryogenesis using whole mount RNA in situ hybridization were unsuccessful (Cannon, Litman and Yoder, data not shown; Thisse and Thisse 2004). These observations are not surprising based on relative levels of expression seen here and noted in previous efforts to recover \textit{nitr}3 cDNAs in library screening. The role of the maternal and embryonic NITR transcripts is unknown but of considerable interest.

The expression of NITR genes in zebrafish lymphoid and myeloid cell populations was characterized by RT-PCR (Fig. 3). Expression of the 14 different NITR gene families was detected in the lymphocyte population, but not in the myeloid population. This expression pattern is consistent with the hypothesis that NITRs are expressed and function in cytotoxic NK-like and T cells in bony fish (Yoder 2009); however, expression of a single flounder NITR and a single catfish NITR have been reported in B cells (Piyaviriyakul et al. 2007; Evenhuis et al. 2007). Although the expression of
certain catfish NITRs in a macrophage cell line (Evenhuis et al. 2007) may be a derived feature of catfish, it may be that some zebrafish NITRs are expressed in myeloid cells, but at a level too low for detection by our assay.

In order to confirm the identity of the NITR amplicons (Figs. 2 and 3) at least one amplicon was sequenced and confirmed to be the predicted gene (data not shown). In an effort to evaluate the ability of the degenerate primers to amplify multiple genes and alleles, multiple nitr1a/b/c/f/k, nitr1d/g/h/i, nitr2a/b/d/e, and nitr3b/d amplicons were sequenced from various cDNA sources. The peptide sequences encoded by these amplicons is provided in Supplemental Fig. 1 and summarized in Fig. 4. Of the 44 nitr1a/b/c/f/k amplicons sequenced from five different cDNA sources, 27 different alleles were identified (different alleles defined as encoding different peptide sequences) including 14 new alleles. Similar results were observed for nitr1d/g/h/i, nitr2a/b/d/e, and nitr3b/d amplicons. In the course of developing rational priming strategies for the nitr1, nitr2, and nitr3 multi-gene families, we had the occasion to examine the allelic variation of nitr5, which represents a single copy gene. Of the 27 nitr5 amplicons sequenced from four different cDNA sources, seven new nitr5 alleles were identified (Supplemental Fig. 1 and Fig. 4). These data further document the polymorphic nature of the NITR gene cluster (Yoder et al. 2004; Yoder 2009) and support a recent and rapid evolution of the NITR gene family.

The work described herein documents that zebrafish NITRs are expressed in the spleen, kidney and intestine; some NITR genes are expressed less abundantly in the liver. The expression of all NITR genes in the lymphoid lineage supports their role as NKRs, as does their high degree of allelic variation (e.g., nitr1, nitr2, nitr3, and nitr5). As a large diversified multigene family, zebrafish NITRs exhibit differential, variable expression in the ovary and during embryogenesis. The nitr3 family is unique in that it is expressed throughout development and suggests that certain NITRs may exhibit alternative functions outside of the leukocyte lineage and their likely primary role as NKRs.

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