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Overexpression of a fish CDKN2 gene in a hereditary melanoma model

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The fish genus Xiphophorus provides a vertebrate model useful in etiological studies of cancer. Hybrid fish can spontaneously develop melanomas deriving from the inheritance of melanistic pigment patterns and the simultaneous absence of proper genetic regulation. A cyclin-dependent kinase inhibitor gene, termed CDKN2X, was mapped to a genomic region that is implicated in fish melanoma tumor suppression. The related human tumor suppressor locus CDKN2A (P16, INK4A, MTS1) is deleted, mutated or transcriptionally repressed through methylation of cytosine bases within the 5′ CpG island in a variety of neoplasms, including melanoma. The fish CDKN2X locus harbors a CpG island within its promoter and first exon, analogous in location to CpG islands in human CDKN2A and CDKN2B loci. The methylation state of individual CpG dinucleotides was investigated in genomic DNA derived from control tissues and melanomas within the CDKN2X 5′ CpG island. The studied genomic area was found to be virtually unmethylated in all tested tissues including melanomas. In addition, RNA expression studies of the fish CDKN2X locus revealed that it is significantly overexpressed in melanoma, in contrast to what has been reported for the human CDKN2A locus in melanoma. Such overexpression may be a consequence of the pronounced upregulation of the Xmrk-2 receptor tyrosine kinase oncogene reported in several Xiphophorus melanoma models.

Introduction

The poeciliid fish genus Xiphophorus has been used for decades as a genetic model for the study of melanoma formation. Many of the 22 described species are polymorphic for sex-linked pigment patterns which are predominantly comprised of large melanin-containing cells called macromelanophores and their precursor melanocytes (1,2). An aberrantly regulated receptor tyrosine kinase gene called Xmrk-2 (3–5) has been mapped to this sex-chromosomal region harboring the macromelanophore locus and is implicated in fish melanomagenesis (3–9).

Numerous fish macromelanophore pigment patterns can become phenotypically enhanced in interspecific hybrids. Severe phenotypic enhancement within select hybrids often leads to nodular melanoma formation (10–13). One well-studied genetic cross (Figure 1) is generated when a Xiphophorus maculatus individual homozygous for a spot sided pigment pattern (called Sp) is mated to a Xiphophorus helleri individual devoid of such a pigment polymorphism (13–16). X.maculatus contributes the Xmrk-2 oncogene which is associated with Sp, while the non-pigmented X.helleri lacks both the Xmrk-2 oncogene and Sp. The resulting F1 hybrids phenotypically show enhancement of the Sp pigment pattern. After backcross to Xiphophorus helleri, half of the animals do not inherit Sp and consequently do not develop macromelanophore cells. Among the progeny that inherit Sp, all animals exhibit phenotypic enhancement of Sp, with some individuals exhibiting severe melanosis and nodular melanomas while others show less extreme phenotypic enhancement similar to that exhibited in the F1 hybrid progenitors. Melanotic tissues within F1 and first generation backcross (BC1) hybrid fish exhibit overexpression of the Xmrk-2 oncogene (17). Studies of the Xmrk-2 oncogene in other Xiphophorus crosses have also shown that it is extremely overexpressed within melanomas and melanotic tissues, and generally mediates proliferation in melanocytes (3–7).

The overexpression of the Xmrk-2 oncogene can therefore be hypothetically viewed as a primary event in Xiphophorus melanomagenesis.

The segregation of ‘heavily’ and ‘lightly’ pigmented individuals in the backcross generation can be genetically explained by a model based on inheritance of an autosomal regulatory factor termed DIFF (13,14,18–21) which modulates the phenotypic expression of the pigment pattern and acts as a melanoma suppressor gene. According to this model, X.helleri either harbors a non-functional (i.e. fails to function properly in macromelanophore cells) DIFF copy (depicted as DIFFh in Figure 1) or lacks this gene altogether (13,20,21). The DIFF locus was mapped to Xiphophorus linkage group V (LG V) (19) and this location has been confirmed by other studies (14,20,22,23).

Human CDKN2A (P16, INK4a, MTS1) and CDKN2B loci are homo- or heterozygously deleted, mutated or transcriptionally repressed in numerous human neoplasms (24–27). The CDKN2A locus, in particular, has been implicated in human melanomagenesis, as mutations/deletions have been described in familial melanoma (28–34), and sporadic melanoma (35–40). In addition, aberrant methylation of the 5′ CpG island of CDKN2A has been reported for a number of melanoma samples (41,42), although this mechanism does not seem to be as prevalent in this cancer as in those of the breast, bladder, colon and prostate (43,44). The human CDKN2B locus has also been shown to be aberrantly methylated at its 5′ CpG island in gliomas and leukemias (26,45,46).

A cyclin-dependent kinase inhibitor gene termed CDKN2X was cloned from Xiphophorus and shows sequence homology to all four identified mammalian gene family members (17,21). CDKN2X codes for a 13 kDa protein which is comprised of nearly four ankyrin repeats, with a genomic structure including only two exons, similar to mammalian CDKN2B (P15, INK4b, MTS2) loci (17). This locus maps to Xiphophorus LG V within a genomic region that has been implicated in both pigment pattern enhancement and tumor formation in backcross hybrid fish (20,21).
Experimental animals and genomic DNA isolation followed by nested amplification of Xmrk-2 external nodular melanoma.

Materials and methods

Investigation, which proved to be significant in obtaining robust PCR amplification. In addition to these measures, we typically employed nested PCR strategies. For PCR amplification of the DNA α-stand, we used primers METFα (5'-agagtgtgtgtgtgatgtgtgtg-3') and METRα (5'-ctcactactacactaccattacc-3') followed by nested amplification using METF4α (5'-tagtgataaatatagttttaa-3') and METR4α (5'-aatcaaacaaacacaaaataaat-3') when necessary. Primers were similarly designed for the β-stand with initial amplification using βMETF1 (5'-ctctactactacactacattat-3') and βMETR1 (5'-agagtgtgtgtgtgatgtgtgtg-3') followed by β-amplification with βMETF3 (5'-tagtgataaatatagttttaa-3') and βMETR3 (5'-aggagtgtgtgtgtgatgtgtgtg-3'; see Figure 2 for overall topology, primer positions and approximate size of amplification products).

All cloning of PCR products was performed with the aid of a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). DNA sequencing was performed with
using a 94°C denaturation for 2 min followed by an amplification parameter of 32 cycles of 94°C for 1 min, 55°C for 1 min, 74°C for 1.5 min. A final incubation of 10 min at 72°C was later followed by a 31°C hold. This product was cloned using the TA cloning kit (Invitrogen) and later subcloned into the EcoRI site of pBluescript (KS'; Stratagene, La Jolla, CA), sequenced (see above) and transcribed in vitro using a Megashortscript kit (using the T7 primer; Ambion, Austin, TX).

Quantitative competitive RT–PCR was performed by adding 49.5 fg of the above-mentioned control RNA to 1 μg of DNase-treated ( Gibco BRL) total RNA. First strand cDNA synthesis was performed with 2 μM P16R18 (5'-tcagcgacgaacaacatcgct-3') using Superscript II and conditions outlined by the manufacturer (Gibco BRL). After RNase H treatment, PCR was performed using P16F16 and P16R18 primers (same conditions as used to create the internal control, above). Products of 368 and 288 bp corresponded to amplification from tissue or control RNAs, respectively. Products were electrophoresed on 1.8% agarose/1X TBE, stained with ethidium bromide to 0.5 μg/ml, and visualized with a IF-500 gel documentation system (Alpha Innotech, San Leandro, CA). The relative UV fluorescence of the products was determined with the aid of NIH Image software (version 1.62). The conditions for quantitative RT–PCR were determined empirically, by use of varying amounts (198, 99, 49.5, 24.8, 12.37, 6.18, 3.09, 1.54, 0.77 and 0.38 fg) of control RNA template to insure that the assay was within a linear range of amplification. Statistical analysis was performed using t-test mean comparisons with SPSS software (SPSS Inc., Chicago, IL).

**Computational methods**

CpG island boundaries, calculations of CpG/GpC ratio and %G-C were performed using the MacCPGSearch (version 1.2) computer program generously provided by James Stroud and John McCarrey (Southwest Foundation for Biomedical Research, TX) (49). MacCPGSearch was used to scan DNA sequences along sequential 100 bp ‘windows’, and to plot averaged CpG/Gc ratios and G+C% values. This program was also used to determine CpG island boundaries (>0.6 CpG/GpC and ≥50% G+C content)(50,51).

**Results**

*Methylation status within the 5’ CpG island of CDKN2X*

The CDKN2X locus of *Xiphophorus* comprises only two exons (17), similar to the CDKN2B locus of human (52). CDKN2X harbors two CpG islands (50,51) encompassing part of the promoter area/first exon as well as part of the second exon (Figure 2). The position of the fish CDKN2X CpG islands is qualitatively similar to those within human and rodent CDKN2A and CDKN2B genes (26,43,44).

Within the first CDKN2X CpG island, 33 CpG dinucleotides exist within a span of 538 bp, distributed within the promoter and the first exon (Figure 2). Since no prior knowledge existed regarding the methylation status of this CpG island, we decided to use a bisulfite genomic DNA treatment (48), followed by PCR amplification (of both α and β DNA strands; Figure 2 and above) and sequencing of numerous individual clones to completely assay for methylation at all cytosine positions. This methodology, although laborious, allows for a quantitative assessment of methylation for all cytosine positions within a studied area. Examination of individual clones is particularly important in melanoma samples since a degree of heterogeneity in melanocytic cell type and inclusion of other cell types is certain and is difficult to avoid (53 and unpublished observations).

To optimize conditions, we employed a strategy of bisulfite treatment of plasmid DNA, PCR amplification of a smaller region of the plasmid, followed by cloning the derived amplimers and sequencing them individually. The original plasmid had been specifically methylated at three sites using HpaII methylase (within the amplified area); this control methodology allowed assurance of complete cytosine conversion (to uracil) and simultaneous assessment of proper detection of methylated cytosines. We tried 16 and 18 h bisulfite treatment incubations, but concluded that these conditions were inadequate and settled.

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**Fig. 2.** Analysis of the distribution of CpG dinucleotides within the *X.helleri CDKN2X* gene. Moving averages of CpG/GpC ratios and G+C% were calculated as described in Materials and methods. Each point on the graph represents the average value for 100 nucleotide positions using sequential ‘windows’ of 100 bp. Values for CpG/GpC are plotted as a solid line while %G+C is plotted as a dotted line. The CpG and GpC densities are plotted for the entire genomic region, with each dinucleotide indicated by vertical lines. The overall topology is depicted, with open boxes depicting transcribed and translated regions, and shading to indicate areas that are only transcribed, as determined experimentally (17). The positions of the two CPG islands (50) with pertinent statistical values are indicated. The 5’ CpG island is enlarged to show the position of individual CpG dinucleotides, exon position and the name and position of primers used in the PCR to amplify α and β DNA strands. The sequence used in this figure was obtained as noted previously (17; GenBank accession no. AF132500). The physical map of *X.maculatus CDKN2X* and the position of CpG islands within it are similar and are not depicted (17; GenBank accession no. U69275).

**RNA isolation and competitive RT–PCR**

Total RNA was extracted with the aid of Trizol reagent ( Gibco BRL, Rockville, MD). For quantitative competitive RT–PCR, an internal control was constructed by first synthesizing cDNA derived from *X.helleri* gill tissue employing a Superscript II pre-amplification kit and the provided Oligo(dT) primer (Gibco BRL, Rockville, MD). A 288 bp RT–PCR amplimer with an internal 70 bp deletion was generated using an established protocol (41) with primer P16F16 (5’-ttgagctgatagcgagc-3’) and a composite downstream primer P16RQ1 (5’-tcagcgacgaacaacatcgct-3’). The PCR was performed with a Omn-E thermal cycler (Hybaid Ltd., UK)
on a 40 h protocol. Ten plasmids, encompassing 8,776 kb of sequence, were fully sequenced to develop the appropriate protocol for eventual employment using genomic DNAs from fish tissues (data not shown).

After optimization of conditions, we determined the methylation status of DNA isolated from normal tissues (gill and liver), derived from X. helleri, X. maculatus, an F1 hybrid and two backcross hybrid fish. Melanoma samples were also examined from the hybrid fish. Multiple clones were derived from each tissue type and individual, and we performed sequencing for both α and β DNA strands to exclude possible differential methylation between DNA strands. These data are summarized in Table I.

For each tissue type, at least four independent clones of PCR products were sequenced; for melanomas this minimum number was raised to nine. A total of 88 plasmids were sequenced comprising 58,371 nucleotides. Within these sequences, 248 misincorporations occurred resulting in an overall error rate of 0.42%, in agreement with error rates reported for non-proofreading thermostable polymerases (54). In addition, only 10 cytosines of 10,645 (0.09%) that lie outside of CpG dinucleotides were not converted to uracil. Therefore, the remaining cytosines found in CpG dinucleotide positions reliably reflect the degree of 5-methylcytosine content in the original genomic DNA material.

The results depicted in Table I indicate that the studied CDKN2X genomic region is almost totally unmethylated. No pronounced differences could be detected between samples derived from melanomas and normal tissues of individual fish, or between hybrid and non-hybrid controls.

**RNA expression of Xiphophorus CDKN2X**

To assess the transcriptional characteristics of CDKN2X, we established and employed a semi-quantitative, competitive RT–PCR assay. The procedure involved use of an in vitro transcribed RNA derived from CDKN2X with an internal deletion of 70 bp that was used as an exogenous control or ‘spike’. This control RNA molecule was added to each total RNA preparation and controlled for the subsequent production of cDNA and amplification with the PCR. Numerous control reactions were analyzed using varying amounts of control RNA and tissue-derived RNA to establish amplification conditions within a linear range of tissue/control amplimer ratio (Figure 3a; see also Materials and methods).

Analysis of CDKN2X RNA expression in X. helleri and X. maculatus revealed detectable expression in all tested tissue samples (Figure 3b). Expression was highest in gill, dorsal fin and tail fin tissues while analysis of muscle, brain, eye and kidney showed moderate expression. The use of liver and heart total RNA revealed barely detectable amplification of the tissue-specific amplimer and so these tissues are considered to weakly express CDKN2X (data not shown). CDKN2X is also expressed in gill, skin and tail fin within tissues derived from BC1 hybrids. In these same hybrids, analysis of CDKN2X expression within melanomas revealed robust expression (Figure 3b). Melanomas showed CDKN2X expression 11 times greater than gill, 82 times greater than skin and 16 times greater than tail fin samples. The difference between melanomas and these control tissues is highly significant (pooled control tissues; two-tailed t-test, \( P = 0.003 \)). In addition, comparison of expression levels within melanomas is significantly greater than non-neoplastic tissues derived from the progenitor species X. helleri and X. maculatus (pooled control tissues; two-tailed t-test, \( P = 0.003 \) and \( P = 0.0001 \), respectively).

**Discussion**

Numerous examples of CpG island methylation of promoter regions being correlated with transcriptional inactivation have been noted previously (55). When such aberrant methylation patterning involves tumor suppressors such as the retinoblastoma (Rb) or Von Hippel–Lindau (VHL) gene products, the consequences of transcriptional repression can contribute to the process of cellular neoplastic transformation (55). In other cases, hypomethylation within genomic regions such as oncogene promoters can result in the activation of such genes and can also contribute to neoplastic transformation (55). In the Gordon–Kosswig Xiphophorus melanoma model, both Xmrk-2 and DIFF are involved in melanoma formation; it has been reported that the Xmrk-2 oncogene is hypomethylated in fish melanomas (53), with the possible consequence of pronounced overexpression documented in several reports (3–5.7). In this study, we have examined the methylation status of the CDKN2X CpG island located within the promoter/first exon region of this candidate gene for the DIFF tumor suppressor.

The fish CDKN2X CpG islands are positionally analogous to those of human and rodent CDKN2A and CDKN2B genes (26,43,44), although the GC content of the fish islands is dramatically lower. As an example, consider the human CDKN2A CpG island within the promoter/exon 1α region, which contains a 0.81 CpG/GpC ratio and a 70.5% G+C content, compared with the 5′ CpG island of CDKN2X which shows a 0.98 CpG/GpC ratio in a genomic area only 51.9% G+C. However, lower G+C content in fish CpG islands has been reported for the trout metallothionein B, carp β-actin and the

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Table I. Methylation of the Xiphophorus CDKN2X promoter

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CDKN2X methylation status and expression

Surfeit genes of puffer fish (56,57). Although CpG islands within these genes were not extensively screened to quantitate the degree of cytosine methylation, experiments using methylation sensitive and insensitive isoschizomers suggested that more numerous (see ref. 24 for review). While examination of the Xiphophorus Xmrk-2 oncogene promoter area, where 84.6% of CpG dinucleotide positions from control tissues contain methylated cytosines (53). Curiously, examination of the studied Xmrk-2 promoter area (53,59) using CpG island criteria of ≥0.6 CpG/GpC and ≥50% G+C reveals that the region could also be defined as harboring a CpG island as well (O/E CpG = 0.68, %G+C = 51.4%). Such intriguing results in Xiphophorus in addition to data presented from researchers using trout, carp and puffer fish cumulatively imply that a revised definition of CpG island criteria is in order for fishes, and further research needs to identify DNA sequences that possibly are important in defining areas of hypo- or hypermethylation.

Aberrant methylation of the 5’ CpG island of CDKN2A has been reported for a number of human melanoma samples (41,42) although this mechanism does not seem to be as prevalent in this cancer as in those of the breast, bladder, colon and prostate (43,44). The human CDKN2B locus has also shown to be aberrantly methylated at its 5’ CpG island in gliomas and leukemias (26,45,46). The comparisons between normal tissues and melanomas from X.helleri, X.maculatus and hybrid fish failed to reveal any differential methylation patterning. This result contraindicates methylation-induced reduction of CDKN2X RNA expression in Xiphophorus melanoma.

Analysis of CDKN2X RNA expression characteristics in non-hybrid fish reveals detectable levels in all tested tissues, although liver and heart showed very low expression. Examination of gill, skin and tail fin samples in BC1 hybrids revealed similar levels of expression. Analysis of melanoma samples revealed an extreme elevation of CDKN2X expression; these levels of transcription significantly differ from control tissues derived from the same BC1 hybrids and from normal tissues derived from X.helleri or X.maculatus. Recently, we have also performed ‘relative’ RT–PCR that quantitates CDKN2X expression versus endogenous 18S rRNA. These data also confirm gross overexpression of CDKN2X in melanoma (17).

The marked overexpression of CDKN2X in fish melanomas parallels the CDKN2A(P16) overexpression found in several neoplastic tissues including a subset of human malignant breast carcinomas (60), cervical and genital lesions (61,62), ovarian carcinomas (63) and mouse bladder carcinomas (64). These reports, however, represent exceptional situations, as references describing lack of CDKN2A(P16) expression due to somatic deletion, mutation or transcriptional repression by promoter cytosine methylation within many other cancers are much more numerous (see ref. 24 for review). While examination of CDKN2A(P16) within familial melanoma pedigrees and individuals developing sporadic melanomas has revealed deletion, mutation or transcriptional silencing, marked overexpression within melanoma cells has not typically been observed (see refs 24,65 for review), in contrast to the fish CDKN2X expression characteristics. This difference may, however, simply be a consequence of the pronounced and consistent overexpression of the Xmrk-2 receptor tyrosine kinase in the fish melanoma model system. Hypothetically, CDKN2X could...
be attempting to regulate the G1 checkpoint phase of the cell cycle and the pronounced proliferation signals of the Xmrk-2 oncogene; such a role is certainly parallel to that of the mammalian tumor suppressor CDKN2A in human cancers, including melanoma, where alterations to critical genes involved in the G1 phase (such as cyclin D1, CDK4 and/or CDKN2A) have been noted (24,36,65).

We have recently documented differential expression of CDKN2X alleles within tissues (including melanomas) derived from heterozygote hybrid fish, as X.maculaus alleles show higher transcript levels than those of X.helleri (17). This quantitative difference may have qualitative consequences that can be manifested in the melanic phenotype. For example, a heterozygote animal (with one allele from X.helleri and one from X.maculaus) may have adequate CDKN2X expression to partially counteract the proliferative effects of Xmrk-2 while a CDKN2X homozygote (two X.helleri alleles) may not (although the expression is still elevated relative to normal tissues). If such a hypothetical scenario is functionally proven, CDKN2X could be defined as being the classically defined DIFF tumor suppressor. Such a relationship between Xmrk-2 and DIFF would imply that DIFF is a downstream regulator of fish melanocyte proliferation/transformation and exclude a hypothesis of DIFF directly regulating the transcriptional properties of Xmrk-2 (an 'upstream' function).

The CDKN2X promoters of X.helleri and X.maculaus have several structural differences and these are currently being assessed for functional consequence. Alternate hypotheses of potential involvement also exist such as possible mutation that have not yet been addressed. In addition, CDKN2X involvement may revolve around the development and differentiation of melanocytes within Xiphophorus embryos. Since BC1 hybrid fry ~7–10 days old display much larger numbers of melanocytes and macromelanophores than non-hybrid fish, it is distinctly possible that CDKN2X allelic differences have already been functionally manifested. To address such hypotheses we are currently initiating studies of CDKN2X within embryos and assessment of immunohistochemical distribution within embryos, fry and adult histological sections. It is hoped that further experimentation will clarify the role of CDKN2X in fish melanomagenesis, and contribute to the overall understanding of melanoma development in vertebrates.

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References


