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Etiology of MNU-Induced Melanomas in *Xiphophorus* Hybrids*

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Abstract

Genetic hybrids of the genus *Xiphophorus* have historically been useful models for study of the genetic aspects of tumor formation. In the most studied *Xiphophorus* tumor model, two gene loci, *XMRK* and *DIFF*, are implicated as critical both to UV-induced and spontaneous melanoma formation in BC₁ hybrids of crosses between *X. maculatus* and *X. helleri*, with *X. helleri* as the recurrent backcross parent. In addition to UV, the direct acting carcinogen N-methyl-N-nitrosourea (MNU) has been used to induce tumors in *Xiphophorus* BC₁ hybrids from several cross types. In the present study, we address the hypothesis that excess melanomas in MNU-treated BC₁ hybrids may have been generated by direct mutation of *CDKN2AB*, a candidate gene for *DIFF*. MNU treatment of F₁ and BC₁ hybrid fish significantly increased tumor incidence at 6 months; however, no association was found between MNU-induced tumor formation and zygosity of the candidate tumor suppressor *CDKN2AB* in BC₁ hybrids, consistent with previously reported results. Sequence analysis of the *X. maculatus* *CDKN2AB* locus of heterozygous individuals (both BC₁ and F₁ hybrids) did not reveal any mutations caused by MNU, suggesting that the mechanism of MNU-induced melanoma formation in this *Xiphophorus* model does not involve direct mutation of *CDKN2AB* but may result from mutation of other critical genes.

Keywords

Xiphophorus; MNU; melanoma; *CDKN2AB*

Introduction

Fishes of the genus *Xiphophorus* have historically been useful models for the study of genetic aspects of melanoma formation. Interspecies *Xiphophorus* hybrids are often employed in these studies due to the increased susceptibility of hybrid offspring to various tumors (Gordon, 1931; Anders et al., 1979; Scharl, 1995; Nairn et al., 1996a). Studies of hybrids from these genetic crosses have yielded important information about both spontaneous and induced melanoma development (Vielkind et al., 1989; A. Scharl et al., 1997). The sex-linked pigmentation patterns in *Xiphophorus* fishes consist of the precursor melanocytes and large

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melanin-containing cells termed macromelanophores that are either enhanced or suppressed depending on the cross performed (Humm and Young, 1956). One such cross between *X. maculatus* Jp 163 B with a spotted-side (Sp) pigmentation pattern and *X. helleri* sarabia strains generates pigmented backcross hybrids that have been well studied for UV induction of melanoma. In this instance, backcross progeny develop melanomas if treated with UV shortly after birth (Setlow, et al., 1989; Nairn, et al., 1996b). The inheritance of melanoma susceptibility is linked to the segregation of a single autosomal gene (termed *DIFF*) with inheritance of the sex-linked pigment pattern locus (*XMRK*-Sp) (Anders, 1991; Scharl, 1995; Kazianis et al., 1998). Progeny of the initial cross (F_1 hybrids) are heterozygous for both loci and show an enhancement of the *X. maculatus* Sp phenotype. When these F_1 progeny are backcrossed to *X. helleri*, half do not inherit the Sp locus and consequently do not develop a pigmentation pattern. The remaining half are heterozygous for Sp and develop either a heavy or light pigmentation pattern depending on the zygosity of their inherited *DIFF* allele (Nairn et al., 2001). Thus the susceptibility of hybrid offspring to melanoma formation can be described by this two-gene model involving *XMRK* and *DIFF*.

The *XMRK* locus encodes an oncogenic receptor tyrosine kinase-related gene, which acquired a different promoter during a gene duplication event, resulting in altered transcriptional control specific for pigment cells (Adam et al., 1993). Two amino acid changes in the *XMRK* protein occurring after gene duplication have resulted in ligand independent and constitutively active signaling (Gomez et al., 2004) leading to induction of both Ras-Raf-MAPK-dependent and independent pathways, resulting in unregulated cellular proliferation (Meierjohann and Scharl, 2006).

Previous work identified the gene *CDKN2AB*, a *Xiphophorus* homologue to the mammalian cyclin dependent kinase inhibitor (*CDKN2*) gene family, as a candidate gene for *DIFF* based on linkage and QTL analysis (Nairn, et al., 1996b; Kazianis et al., 1998). Sequence analysis of *CDKN2AB* alleles from *X. maculatus* and *X. helleri* revealed only two amino acid differences suggesting that there were no functional differences between the proteins encoded by these two alleles; however differential levels of transcription from *X. maculatus* and *X. helleri* alleles were detected, with the *X. maculatus* allele overexpressed more than 11-fold in BC_1 heterozygotes. Additionally, backcross progeny homozygous for the *X. helleri* *DIFF* allele develop the heavily pigmented phenotype and are more likely to develop melanomas than heterozygous individuals (Kazianis et al., 1999). In the two-gene model for inheritance of melanoma susceptibility, *DIFF* would act downstream from *XMRK* as a tumor-suppressor gene with the *X. maculatus* and *X. helleri* alleles expressing *CDKN2AB* (candidate for *DIFF*) at different levels. In heterozygous hybrids, the higher expression from the *X. maculatus* allele may be sufficient to compensate for the proliferation signals driven by *XMRK*. However in homozygous BC_1 individuals, which inherit only the *CDKN2AB* from *X. helleri*, the reduced expression may not fully mitigate the oncogenic signal from *XMRK*, leading to an increased incidence of melanoma in these individuals (Nairn et al., 2001; Butler et al., 2007).

The direct acting carcinogen N-methyl-N-nitrosourea (MNU) has been used in addition to UV to induce melanomas in hybrid fish of the cross described above (*X. maculatus* Jp 163 B with *X. helleri*). Previous work using MNU to induce melanomas in BC_1 hybrids indicated that *XMRK* was a critical genetic determinant of induced melanoma susceptibility. However, unlike the results of experiments with UV-induced tumors, no genetic association was found between *CDKN2AB* alleles and MNU-induced melanoma susceptibility. Tumor incidence in these experiments was about twice that of UV-induced melanomas in the same cross (Kazianis et al., 2001).

In heterozygous individuals, the *X. maculatus* allele would be expected to provide adequate *CDKN2AB* protein to compensate for the lower expression of the *X. helleri* allele, thereby

counteracting the proliferation signals generated by increased expression of *XMRK* (Nairn et al., 2001). However, if MNU treatment causes mutations within the *X. maculatus CDKN2AB* allele that either inactivate it or result in a lowered expression level, the anti-proliferative effect may be eliminated. The work described here aims to confirm the previous result that melanomas are induced by MNU treatment in this genetic cross, and to test the hypothesis that the *X. maculatus CDKN2AB* allele in heterozygous BC₁ individuals might be a target for MNU, causing direct mutations in *CDKN2AB* that may result in the observed increase in tumor incidence in MNU-treated hybrids. Such a direct mutational mechanism may be masking any genetic association that would otherwise be revealed by linkage analysis.

Materials and methods

Generation of hybrid offspring and MNU exposure

Hybrid offspring were generated by the following crossing scheme (Figure 1): Female *X. maculatus* of strain Jp 163 B bearing the “spotted side” (Sp) pigment pattern were artificially inseminated with sperm collected from *X. helleri* males. F₁ hybrid offspring were collected and backcrossed to *X. helleri* to generate backcross (BC₁) progeny. Five week old F₁ and BC₁ hybrid offspring were treated with 1 mM MNU (Sigma, St. Louis) for one hour as described previously (Kazianis et al., 2001). This exposure was repeated every other day for a total of 4 treatments. UVB tumors were induced as described by Nairn et al. (1996b), then excised and quick frozen until use.

Tumors from both treated and untreated fish were scored at 6 months or earlier and were allowed to progress until they were large enough to yield sufficient tissue for analysis. Tissues were removed and immediately frozen on liquid nitrogen then kept at −80°C. Selected tumor tissues were fixed in 10% buffered formalin and embedded in paraffin for histological examination.

DNA isolation, genotyping and genetic analysis of *CDKN2AB* allele

DNA was isolated from frozen gill samples taken from tumor-bearing hybrid fish using the Puregene kit (Gentra Systems, Minneapolis, MN) and the genotype of each BC₁ fish was determined at the *CDKN2AB* locus using a previously described PCR-based assay (Kazianis et al., 1998). DNA was then isolated from tumors of heterozygous BC₁ individuals. Two regions encompassing the two exons of the *X. maculatus CDKN2AB* allele were amplified by PCR using the primer sets p16S3 (TGTCGCCGACAATGACCG) + p16AS1 (TGCCTTGTCTGCCAGTTATTACC) with a 68°C annealing temperature yielding a 331 bp product and p16S2 (GTAGTAGTGCAATAGCGAGACATAAA) + p16AS2 (GCCTTATTCACGGTTCTCAATCAG) with a 66°C annealing temperature yielding a 542 bp (Figure 2). Each PCR product was purified (Promega SV PCR cleanup kit) and cloned using the Invitrogen TA cloning system. Six independent clones were picked from each reaction, grown up and DNA was prepared using the Promega Wizard Plus Miniprep system. After confirming that all clones contained the appropriately sized insert, each was sequenced with the T7 primer contained within the TA cloning vector using an ABI Prism 3100 DNA Sequencer and the BigDye Terminator kit (ABI). All sequences were aligned and analyzed using MacVector software (MacVector, Inc. Cary, NC).

Results

The present work was performed for the purpose of validating previously reported results regarding the induction of melanomas by MNU in offspring of a specific *Xiphophorus* cross (Kazianis et al., 2001), as well as to test the hypothesis that the *X. maculatus CDKN2AB* allele in heterozygous BC₁ individuals might be a target for MNU. During the course of this study

176 F₁ hybrid and 401 BC₁ hybrid offspring of the *Xiphophorus* cross depicted in Figure 1 were treated with MNU yielding 14 and 87 cases of melanoma respectively (Table 1). Melanomas were only observed in offspring inheriting the Sp pigmentation pattern, consequently incidence was only calculated for Sp-bearing fish. Untreated control F₁ (186 individuals) and BC₁ (230 individuals) fish were also maintained and monitored for melanoma development. A subset of melanomas was sampled for histology and representative examples are shown in Figure 3 where the infiltration of the fish musculature by melanoma cells is clearly evident. The incidence of melanoma formation was significantly elevated in both groups of hybrids treated with MNU when compared to control groups (Table 1). However, the difference in incidence was more dramatic in BC₁ hybrid individuals (7.95% in F₁ hybrids compared to 21.7% in BC₁ hybrids).

The increased incidence of MNU-induced tumor formation among BC₁ fishes was hypothesized to be due to the activity of the two different *CDKN2AB* alleles (*X. maculatus* vs. *X. helleri*) present in BC₁ offspring. Previous work (Nairn et al., 1996a, 1996b) has shown that spontaneous and UV-induced melanoma formation was correlated with the homozygous *CDKN2AB* genotype where tumor-bearing individuals were more likely to be homozygous for the *X. helleri* allele. In order to examine this association in the case of MNU-induced melanoma formation, gills from tumor bearing BC₁ individuals were used to establish the genotype of each fish at the *CDKN2AB* locus. Unlike the data for spontaneous and UV-induced melanoma, no association between melanoma formation and *CDKN2AB* genotype was found when fish bearing MNU-induced tumors were analyzed (Table 2). Our results confirm those of Kazianis et al., (2001) who also reported lack of a genetic linkage in MNU-induced tumors.

It was previously hypothesized that a direct mutation in the *X. maculatus CDKN2AB* allele might be masking any genetic association between tumor formation and genotype (Kazianis et al., 2001). Such a direct mutation within the *X. maculatus CDKN2AB* allele could lead to an excess of melanomas in heterozygous individuals. Direct sequencing of two regions encompassing the two exons of the *X. maculatus CDKN2AB* allele was performed in order to test for such direct mutations. Both MNU and UV-induced tumors as well as spontaneous tumors were sequenced but no mutations were found among any of the groups analyzed (Table 3) suggesting the mechanism of MNU-induced melanoma formation does not involve direct mutation of the candidate tumor suppressor gene *CDKN2AB*.

Discussion

The *Xiphophorus* fish model is an excellent example of an inducible model of cancer and study of this model has been important for understanding the impact of UV irradiation on melanoma formation in humans (Setlow et al., 1989, 1993). In this study, we confirmed (with a larger sample size) previously reported results (Kazianis et al., 2001) describing the induction of melanoma using the direct-acting carcinogen MNU on progeny from the UV-inducible *X. maculatus* Jp 163 B and *X. helleri* cross. In our study, the BC₁ offspring of this cross exhibited a 3.5-fold increase in MNU-induced melanoma susceptibility compared to untreated individuals. These results suggest a genetic component to the induction of melanomas by MNU whereby backcross progeny are more likely to develop melanoma than parental or F₁ generation offspring.

Previously performed linkage analysis of UV-induced melanomas suggested an association between tumor formation and the *DIF* candidate tumor suppressor *CDKN2AB* genotype where BC₁ individuals homozygous for the *X. helleri* allele were more likely to develop melanomas than their heterozygous counterparts (Nairn et al., 1996a; 1996b). However, when this association was examined in the context of MNU-induced melanomas previously (Kazianis et al., 2001) and again in this study, no significant linkage was found between incidence of

MNU-induced melanomas and *CDKN2AB* genotype. One possible reason for this lack of association, as postulated by Kazianis et al., (2001), was that direct mutations within the somatic *X. maculatus CDKN2AB* gene may be masking the linkage association by making heterozygous (one copy each of *X. maculatus* and *X. helleri CDKN2AB* alleles) individuals as likely to develop melanoma as those with two *X. helleri* alleles. Such a mutation could inactivate the *X. maculatus* allele resulting in functional expression of *CDKN2AB* from only the *X. helleri* allele. However, after sequence analysis of two regions containing the entire coding sequence of the *X. maculatus CDKN2AB* allele in heterozygous BC₁ fish with UV and MNU-induced tumors, no such mutation was found, suggesting that a somatic mutation within the *X. maculatus CDKN2AB* allele was not the cause of the increased tumor incidence in BC₁ hybrids.

It is hypothetically possible that a promoter mutation, or a mutation in the untranslated region (UTR) of the *X. maculatus CDKN2AB* allele, rather than in the coding sequence, could have been induced by MNU and resulted in inactivation; our analyses would not have detected such events. There are examples of human mutations in *CDKN2A* in which an aberrant initiation codon occurs upstream from the normal translational start site, and suppresses its expression presumably due to competition with the normal AUG (Liu et al., 1999, Harland et al., 2000). However, this is an example of a founder non-coding sequence mutation predisposing to melanoma, and it is extremely unlikely that in our experiments such mutations would have occurred exclusively, that is, in the absence of coding sequence mutations as well.

Previous work demonstrated a positive relationship between the *XMRK* oncogene and *CDKN2AB* expression in melanomas (Butler et al., 2007). A previously proposed hypothetical model explaining this relationship between *CDKN2AB* overexpression and *XMRK*-induced hyperproliferation in UV-induced melanomas depends on differential expression between the two *CDKN2AB* alleles (Nairn et al., 2001) as is observed in heterozygous individuals with UV-induced tumors (Kazianis et al., 1999). In this model, overexpression of the *X. maculatus CDKN2AB* allele relative to the *X. helleri* allele in heterozygous individuals provides sufficient *CDKN2AB* to compensate for the proliferation signals generated by elevated *XMRK* expression and fewer tumors result. In homozygous individuals with two alleles of *X. helleri*, the lower expression of *CDKN2AB* is insufficient to compensate for *XMRK* proliferation signals and tumors develop (Nairn et al., 2001). An analysis of the promoter structure for several *Xiphophorus* species has been performed and has revealed interesting differences, most notably the length of a (GT)_n repeat sequence and the presence of a deletion in the *X. helleri* promoter relative to that of *X. maculatus* (Kazianis et al., 1999). If this differential expression were altered by the action of MNU on the promoter of the *X. maculatus* allele resulting in decreased expression of *CDKN2AB*, an increased incidence of melanomas may result.

An alternative explanation for the increased incidence of MNU-induced melanomas in the BC₁ fish is that MNU is acting on a different genetic target responsible for regulation of pigment cell proliferation or transformation in interspecies crosses. It has been hypothesized that the *DIFF* locus (for which *CDKN2AB* is a candidate) may not solely regulate melanoma formation in the cross used in this study in contrast to co-inheritance of spontaneous melanoma and pigment patterns in the classic Gordon-Kosswig cross (Nairn et al., 1996b). Studies of mammalian systems using MNU to induce tumors in rats have shown that the oncogenes p53 and Ras may be specific targets for MNU-induced activating mutations (Sukumar et al., 1991; Matsumoto et al., 1997). Activating mutations in both p53 and Ras are thought to be important in the development of human malignant melanoma along with mutations in the *CDKN2A* gene (the human homologue for *Xiphophorus CDKN2AB*) (Benjamin et al., 2007). It is possible that MNU mutations in p53 and/or Ras or other genes may be contributing to the increase in MNU-induced melanomas in BC₁ hybrids regardless of the zygosity at the *CDKN2AB* locus; however, in this case an upregulation of Ras would be unlikely to increase

melanoma formation as *XMRK* (a upstream activator of Ras) is already overexpressed in tumors (Schartl, 1990). Additional studies may yield important information about how the genes in this complex model work together to result in the initiation of melanoma.

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Figure 1.

Diagram depicting the crossing scheme used to generate the interspecies backcross hybrids used in this study. An *X. maculatus* homozygote for a spot sided pigment pattern (*Sp*) is crossed with a *X. helleri* lacking this pigmentation pattern ($+/+$). The resulting F₁ hybrid individuals exhibit an enhancement of the spot sided pigment pattern and are heterozygous for both the *Sp* and *DIFF* locus. Male F₁ hybrids are further crossed with female *X. helleri* to generate first-generation backcross (BC₁) hybrids, which exhibit a Mendelian distribution of pigmentation phenotypes. Individuals heterozygous for *Sp* are pigmented to different degrees (heavy or light) as regulated by the *DIFF* gene. Those progeny heterozygous for the *DIFF* gene (one allele *X. helleri*, one allele *X. maculatus*) have pigmentation patterns resembling the F₁ hybrids while those homozygous for the *X. helleri* *DIFF* allele are heavily pigmented and are more susceptible to UV-induced melanomas.

Figure 2.

Diagram of *X. maculatus CDKN2AB* gene locus. Shown is the complete gene (without the 5' UTR) with the locations of the two exons depicted as light gray arrows. The two PCR products used for sequence analysis are shown as dark gray boxes below the two exons. See Materials and Methods for primer sequences.

Figure 3.

Histopathology of melanomas taken from hybrid fish. **A.** and **C.** Representative examples of melanomas taken in cross section through the body of two fish. Note the melanoma infiltrating the muscular structure. 4x magnification. **B.** and **D.** High magnification of the same tumors. Note the muscle bundle compressed by the invasion of the melanoma. 40x magnification.

Table 1BC₁ hybrid melanoma incidence[†]

	Treatment group	Total fish	Melanoma (% incidence)
F ₁ hybrid	Control	186	2 (1.07%)
	MNU	176	14 (7.95%) [*]
BC ₁ hybrid	Control	230	17 (6.1%)
	MNU	401	87 (21.7%) ^{**}

[†] Incidence was calculated for Sp-bearing fish only^{*} Significant difference using Chi square test p<0.005^{**} Significant difference using Chi square test p<0.001

Table 2
Association of melanoma formation with *CDKN2AB* genotype

Treatment	Genotyped fish with melanomas	Heterozygotes	Homozygotes	Recomb. %	lod*
Control [†]	49	8	41	16.3	5.3
UVB [‡]	64	14	50	21.9	4.7
MINU	36	12	24	33.3	0.9

[†] Previously reported data included for comparison (Kazianis et al., 2001)

* A lod value above 3.0 is considered significant evidence for linkage association

Table 3*CDKN2AB* sequence analysis

	No. tumors analyzed *	Total sequence reactions analyzed **	Mutations found
Control BC ₁	3	36	0
UVB treated BC ₁	33	396	0
MNU treated BC ₁	10	120	0
MNU treated F ₁	8	96	0

* Only tumors from individuals heterozygous at *CDKN2AB* locus were analyzed

** Includes both primer sets