Genetical studies on melanomas in *Xiphophorus* fish

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ABSTRACT

Cytogenetic and molecular studies were carried out on melanomas in aquarium fish. Twenty Swordtail fish with melanomas were compared with radiation-induced melanomas by gamma rays and ultraviolet light. Significant increase in the mean percentage of micronuclei was found only in the gamma ray-induced melanomas. On the other hand, both the spontaneous and the radiation-induced melanomas revealed significant increases in the total structural as well as numerical chromosomal aberrations compared with the control group. The RAPD/PCR test, using 10 random primers, showed positive results with two primers only that gave distinctive banding patterns with a high degree of divergence between the control groups and the spontaneous melanomas groups. However, the primers failed to give any fingerprints in the radiation-induced melanomas group. This could be attributed to one of two possibilities: the destructive effect of radiation on the DNA sequence complementary to the primers, or the radiation may produce different melanomas as mentioned in the literature.

Keywords: *Cytogenetic, molecular, melanomas, radiation, fish.*

INTRODUCTION

Swordtail fish are widely used as laboratory animals, especially for carcinogenecity studies. They have also an economic importance for home breeding, decoration and as a source of income in case of exportation to other countries. Breeding of coloured fish has developed greatly in Egypt, and many farms are directed at decorative fish production either for the local or international market. Swordtail fish are considered to be one of the most important fish species reared in Egypt due to the high productivity, different colors and high resistance to different weather changes.

Since 1942, Lucke and Schlumberger have observed similarity of the neoplasms between fish and the higher vertebrates, including man. They stated that the high incidence of tumorigenesis in Swordtails due to inbreeding and/or exposure to radiation caused severe economic losses in aquarium fish farms. The incidence of melanoma has showed an alarming worldwide increase (Rigel *et al.*, 1987 and DeGrajil and Van der Leum, 1993). Heredity, target cell susceptibility and excessive sunlight exposure are all believed to be predisposing factors in the development of coetaneous malignant melanoma (Meyer and Zone, 1994). However, a precise role for each of these factors in melanoma formation has not been established (Koh *et al.*, 1990).

The aim of the present investigation is to study the cytogenetic and molecular aspects in Swordtail fish having spontaneous as well as radiation-induced melanomas, to clarify if there is a relationship between different types of radiation and the development of melanomas.

MATERIALS AND METHODS

Fish and radiation

A sample of 530 fish of the Swordtail species (*Xiphophorus*) with an average body

Asfour Farm for Breeding of coloured Fish, Shabrament, Giza, Egypt. From this sample, 20 fish showed black spots and /or swellings and were suspected to indicate spontaneous melanomas. This suspicion was clarified using a histopathological technique as well as an electron microscope, (Sokkar *et al.*, 2001). Five from these spontaneous melanoma-containing fish were taken for cytogenetic analysis and the remaining 15 were used for molecular studies. Those with spontaneous melanomas were examined in comparison with the normal fish and those with two types of radiation induced melanomas (Gamma rays and ultraviolet light).

To induce melanoma, fish were exposed either to gamma rays or ultraviolet light. A sample of 50 fish was collected from the same farm and divided into five groups, each one contained 10 fish. Group GP 1 (control), GP 2 (2 Gy gamma-rays), GP3 (4 Gy gamma- rays), Gp 4 (6 Gy gamma- rays) and GP 5 (UV light). Each group was divided into two parts : five fish were used for chromosomal analysis and for molecular studies as well, and the other five fish were left for the study of the micronucleus test (MNT).

Radiation of the fish was carried out in the National Center for Radiation Research and Technology, Nasr City, Cairo, Egypt, using Gamma Cell 40, Cesium 137 Irradiation Unit for gamma irradiation, while UV irradiation was carried out using FS 40 sun lamp, filtered to give 380 nm (2400 J/m-dose) Setlow *et al.*,(1993). The fish were exposed to each dose of radiation three times with an interval of three weeks between each exposure.

Chromosomal preparation

The technique of chromosomal preparation of fish samples was applied according to Al- Sabti *et al.* (1983) with some modifications as follows: each live-fish was injected intramuscularly with 0.01 ml colchicine /g body weight for three hours. Fish were then dissected and samples from the anterior part of the kidney (head kidney) were collected. At least 50 metaphase spreads from each specimen were prepared, and a research microscope using an oil immersion lens to examine chromosomes was used.

Micronucleus test

A drop of blood from the gills was mixed with a drop of fetal calf serum on a glass slide, and then air-dried. The specimen was then fixed in absolute methyl alcohol for five minutes, and the slide was stained with 10% Giemsa stain for 10 minutes (Deflora *et al.*, 1993). The data were then statistically analyzed using standard Chi-Square, (Mather, 1957).

Molecular analysis

The genomic DNA was isolated using phenol/chloroform extraction and ethanol precipitation method with some modifications (Sambrook *et al.*, 1989). The purity of the DNA preparation was judged by examining the ratio of absorbency at 260 nm and 280 nm (Aquadro *et al.*, 1992).

RAPD - PCR analysis

Amplification of DNA with 10- mer random primers (10 primers) was conducted according to the method of Plotsky *et al.* (1995). The 50 µl reaction volume contained 100 ng genomic DNA; 100 µM dNTP; 40 nM primer (Operon, Almeda, CA, USA); 2.5 units of Taq DNA polymerase and 5 µl Promega 10X Taq DNA polymerase buffer. The reactions were carried out in (Perkin - Elmer 9700) Thermo-Cycler using the following program: heat-inactivation C (5 min), 45 cycles of denaturing C (1 min), DNA reannealing C (1 min) and extension C (2 min). The PCR product was analyzed by electrophoresing 25 µl of the amplified mixture on agarose gel. The Gel - Pro Analyzer (Media Cybernetics) was used to document ethidium bromide DNA gels.

RESULTS

Micronuclei formation in fish

The percentage of micronuclei of the examined fish erythrocytes which had spontaneous melanomas was 0.3% as shown in Table (1). This percentage showed insignificant difference when compared with the control. The formation of micronuclei in the irradiated fish was summarized in Table (1). The statistical analysis of the data revealed that the exposure of fish to gamma-rays with different doses induced a significant increase in the micronuclei, while the exposure to UV light showed non-significant increase in comparison with the control. The percentage of micronuclei formation was 0.7%, 0.94%, and 1.2% in the fish exposed to 2 Gy, 4 Gy and 6 Gy gamma-rays, respectively; which indicated a highly significant increase when compared with the control (0.18 %) (Fig.1).

Tables (2) and (3) showed the number and mean values of fish cells with chromosomal aberrations in the case of spontaneous melanomas and those induced by different doses of gamma - rays and UV irradiation. The mean value of the total aberrant cells in fish with spontaneous melanomas showed a highly significant increase when compared with the control

Table (1): Percentages and mean values of micronuclei in both spontaneous melanomas and irradiated swordtail fish.

Treatment	No. of MN/ Fish	Total No. of MN	MN	
			%	Mean ± SE
Control	3 2 1 1 2	9	0.18	1.8 ± 0.75
Spontaneous melanoma	4 4 3 3 1	15	0.3	3 ± 1.10
Gamma rays 2 gy.	8 7 5 5 10	35	0.7	7 ± 1.90**
Gamma rays 4 gy.	9 10 11 9 8	47	0.94	9.4 ± 1.02***
Gamma rays 6 gy.	10 12 13 11 14	60	1.20	12 ± 1.41***
U.V. light.	4 4 4 3 3	18	0.36	3.6 ± 0.49

MN = Micronuclei.
No. individuals examined = 5 fish / item
No. cells examined = 1000/ fish
*** Astrics indicate significance

Table (2): Mean ± SE chromosomal aberrations of spontaneous melanomas of swordtail fish.

Type of treatment	No. of Examined Fish	No. of examined cells	Chromosomal aberration													Total Aberrant Cell	No. of cells have more than 1 aberr.
			Structural aberrations									Numerical aberrations					
			Gaps	Chr. gap	Delet & frag.	Break	Chr. break	Ring	C.A	Other	Total	Total without gap	2n + 1 or 2	2n - 1 or 2	Poly-ploid	Total	
Control	5	250	0.4 ± 0.49	0.00 ± 0.00	0.4 ± 0.49	0.00 ± 0.oo	0.2 ± 0.4	0.00 ± 0.00	0.00 ± 0.00	0.4 ± 0.49	1.6 ± 0.49	1.2 ± 0.4	1.4 ± 0.49	0.4 ± 0.49	0.00 ± 0.00	1.8 ± 0.4	3.4 ± 0.49	0.8 ± 0.75
Spontaneous melanoma	5	250	1.4 ± 0.49	0.4 ± 0.49	1.8 ± 1.02	1.00 ± 0.63*	0.8 ± 0.4	0.2 ± 0.4	0.8 ± 0.75*	0.6 ± 0.8	6.6 ± 12**	5.2 ± 1.17***	3.6 ± 1.02*	0.8 ± 0.75	1.4 ± 0.8**	5.8 ± 1.47***	12.4 ± 1.02***	2.4 ± 0.49**

Chr. = Chromosome
C.A. = Centromeric attenuation.
Others = Centric fusion & sticky chromosome.
Polyploid = 3 n , 4 n & 5 n .
Delet. & Frag. = Deletion & Fragment.

* = P < 0.05 (Significant).
** = P < 0.01 (highly significant).
*** = P < 0.001 (highly significant).

Table (3): Mean SE of chromosomal aberrations of experimentally irradiated swordtail fish.

T ype of Treatment	Chromosomal aberration													Total Aberrant	No. of cells Have more
	Structural aberrations									Numerical aberrations					
	Gaps	Chr. gap	Delet & frag.	Break	Chr. break	Ring	C.A	Other	Total	Total without gap	2n + 1 or 2	2n - 1 or 2	Poly-ploid	Total	Cells	Than 1 aberr.
Control	0.4 ± 0.49	0.00 ± 0.00	0.4 ± 0.49	0.00 ± 0.00	0.2 ± 0.4	0.00 ± 0.00	0.00 ± 0.00	0.4 ± 0.49	1.6 ± 0.49	1.2 ± 0.4	1.4 ± 0.49	0.4 ± 0.49	0.00 ± 0.00	1.8 ± 0.4	3.4 ± 0.49	0.8 ± 0.75
Radiation with 2 Gy gamma rays	1.4 ± 0.49	0.8 ± 0.75*	1.4 ± 0.8	0.2 ± 0.4	0.6 ± 0.49	0.00 ± 0.00	0.8 ± 0.75*	0.2 ± 0.4	5.4 ± 12**	4.00 ± 1.41***	2.2 ± 0.75	1.00 ± 0.63	0.6 ± 0.49	3.8 ± 0.75	9.2 ± 1.66***	2.8 ± 0.75**
Radiation with 4 Gy gamma rays	2.00 ± 0.63*	0.8 ± 0.75*	1.8 ± 0.4*	1.00 ± 0.63*	0.8 ± 0.4	1.00 ± 0.63*	0.4 ± 0.49	0.6 ± 0.8	8.4 ± 85**	6.4 ± 1.5***	3.2 ± 0.79	1.8 ± 0.4*	2.2 ± 1.17*	7.2 ± 0.75**	15.6 ± 2.15***	3.6 ± 0.8**
Radiation with 6 G gamma rays	2.4 ± 1.02*	1.8 ± 0.75*	1.8 ± 0.75*	0.8 ± 0.75*	1.4 ± 0.49*	1.6 ± 0.49*	1.6 ± 1.02*	1.2 ± 0.75	12.6 ± 85**	10.2 ± 1.67***	4.00 ± 0.63*	2.2 ± 0.75*	2.8 ± 1.17*	9.00 ± 1.55**	21.6 ± 2.8***	4.6 ± 1.02***
Radiation with UV light	1.6 ± 0.49	1.00 ± 0.00	1.2 ± 0.75	0.2 ± 0.4	0.2 ± 0.4	0.4 ± 0.49	1.00 ± 0.63*	0.6 ± 0.49	6.2 ± 17**	4.6 ± 1.02**	3.2 ± 0.4	1.2 ± 0.75	0.6 ± 0.8	5.00 ± 1.67**	11.2 ± 2.48***	2.8 ± 0.75*
Between 2 4 G gamma rays and ultraviolet light rays	2.00 ± 0.63	0.8 ± 0.75	1.8 ± 0.4	1.00 ± 0.65	0.8 ± 0.4	1.00 ± 0.63*	0.4 ± 0.49	0.6 ± 0.8	8.4 ± 1.85	6.4 ± 1.5	3.2 ± 1.5	1.8 ± 0.4	2.2 ± 1.17*	7.2 ± 0.75*	15.6 ± 2.15**	3.6 ± 0.8
Between 2 6 G gamma rays	2.4 ± 1.02	1.8 ± 0.75	1.8 ± 0.75	0.8 ± 0.75	1.4 ± 0.49	1.6 ± 0.49**	1.6 ± 1.02	1.2 ± 0.75	12.6 ± 85**	10.2 ± 1.67***	4.00 ± 0.63	2.2 ± 0.75	2.8 ± 1.17**	9.00 ± 1.55**	21.6 ± 2.8***	4.6 ± 1.02
Between 4 6 G gamma rays	2.4 ± 1.02	1.8 ± 0.75	1.8 ± 0.75	0.8 ± 0.75	1.4 ± 0.49	1.6 ± 0.49	1.6 ± 1.02	1.2 ± 0.75	12.6 ± 1.85*	10.2 ± 1.41*	4.00 ± 0.63	2.2 ± 0.75	2.8 ± 1.17	9.00 ± 1.55	21.6 ± 2.8*	4.6 ± 1.02

Each item = 5 fish **250 cells / 5 fish** ** Significant *** high Significant

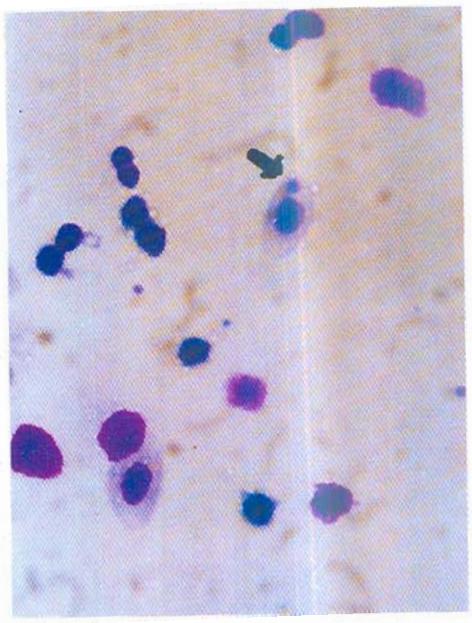
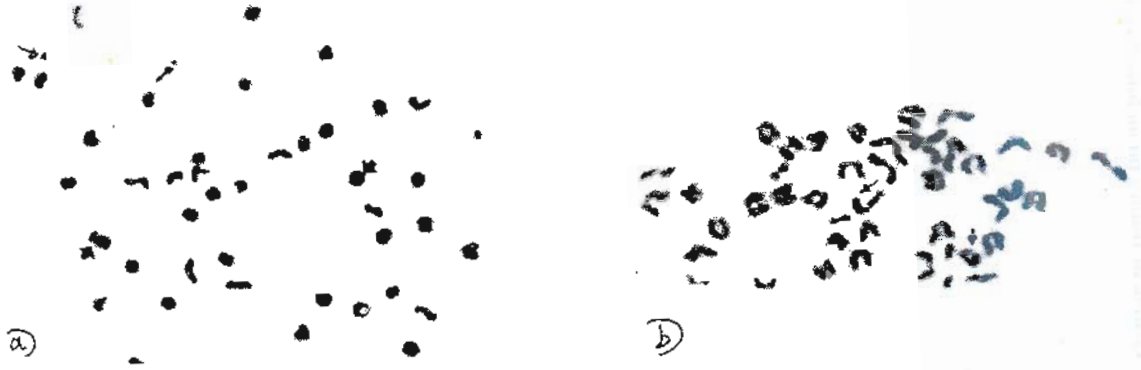


Fig.(1): Blood film of Swordtail fish Showing micronuclei (arrow) X 1000.



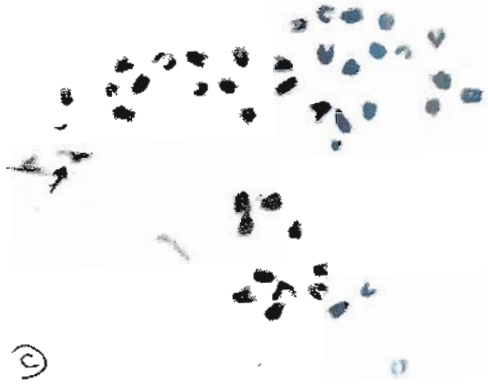


Fig. (2): Metaphase spread of Swordtail chromosomes Showing (X1250), arrows point to:
A) Centric fusion, Ring & fragments
B) Chromosome gap & deletion
C) Chromatid break

(P<0.001). However, non-significant increase was found in the mean value of other aberrations as compared with the control.

The most common types of chromosomal aberrations were chromatid and chromosome gaps, fragments, breaks and ring chromosomes. The total structural aberrations in both gamma-rays (2 Gy dose) and UV irradiated fish showed significant variations when compared with the control (P<0.001). The fish group exposed to 4 Gy gamma-rays revealed more structural aberrations, but the 6 Gy group showed a dramatic increase in both structural and numerical aberrations. In this group, the increase of total structural and numerical aberrations was highly significant with mean values of 12.6±1.85 and 9.00± 1.55, respectively.

DNA fingerprinting

The genomic density of the fingerprints generated by the 10 arbitrary primers used was evaluated among Swordtail fish (liver cell DNA). Fish with spontaneous melanoma were compared with fish that contained radiation-induced melanoma in relation to control fish.

From the ten primers used, only two (OpA-05 and OpA-09) gave positive and detectable fingerprints. By using primer OpA-05, the control fish produced a major sharing band between the individuals which had a size of 210 bp (Fig.3). The DNA of the spontaneous melanoma analyzed with the same primer (OpA-05) revealed the appearance of three other new bands which had the sizes 789, 612 and 458 bp (Fig. 4). Concerning the second positive primer (OpA-09) , the fingerprints obtained are shown in Figs. (3 and 4). The control fish resulted in four sharing bands between its individuals (1,2,3 and 4). The sizes of these fragments were 480, 283, 258, and 140 bp, respectively. It was also noticed that bands 1 and 4 were detected in all individuals tested. The same trend was found in the DNA of spontaneous melanoma using the same primer, but bands number 2 and 3 completely disappeared in all individuals, while new bands (320 and 354 bp) appeared in most of these individuals.

The genomic DNAs of the normal fish samples as well as the irradiated ones were extracted and analyzed using RAPD-PCR technique with the ten primer battery. The data showed that none of the ten primers used, (even with OpA-05 and OPA-09) gave any positive fingerprints.

Table (4): Gel Pro analysis of the RAPD PCR using Primers OpA O5 and OpA O9

Primers	Bands Produced		Primer Sequence
	Control	Tumor	
OpA O5	-	789 bp	5-AGGGGTCTTG-3
	-	612 bp	
	-	458 bp	
	218 bp	218 bp	
OpA O9	480 bp	480 bp	5-GGGTAACGCC-3
	283 bp	354 bp	
	258 bp	320 bp	
	140 bp	140 bp	

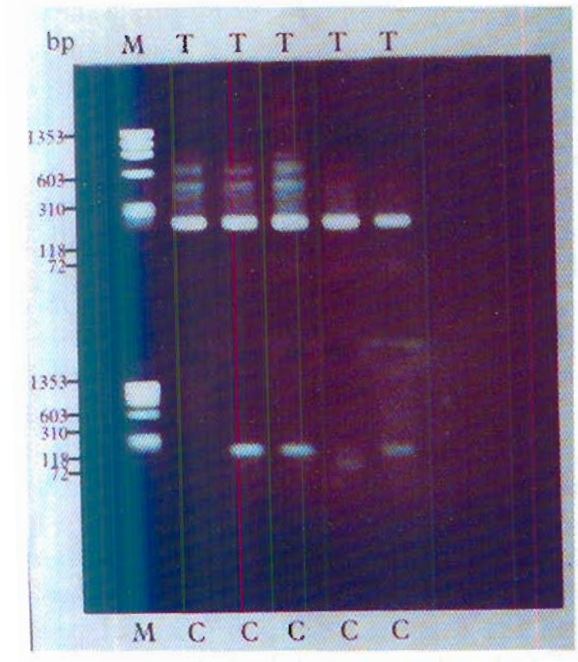


Fig.(3): Gel electrophroresis of the PCR product Of melanoma bearing fish (T) and control fish

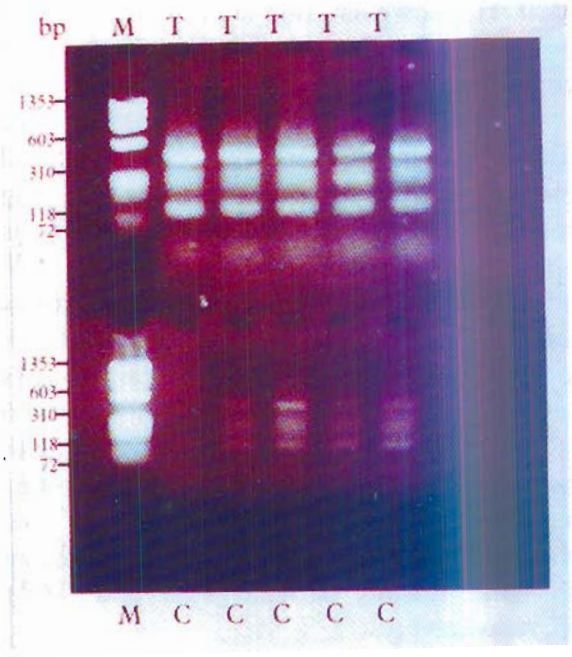


Fig.(4): Gel electrophoresis of the PCR Products of melanoma bearing fish (T) and control fish (C) using OpA-O9 primer M= marker.

DISCUSSION

In this study, the normal karyotype of swordtail (*Xiphophorus*) chromosomes revealed that the normal diploid number is 2 n = 48. Similar results were obtained by Forster and Anders (1977) and Tantithakura (1990). The examination of the fish chromosomes showing spontaneous melanomas revealed structural chromosomal aberrations including breaks and centromeric attenuation in comparison to the control, while the chromosomal numerical aberrations were mainly autosomal trisomics and polyploidy. The presence of gaps and breaks are both indicators of genetic damage and are potentially carcinogenic when occurring in somatic cells (Koller, 1973). On the other hand, most of the structural chromosomal aberrations lead to genetic changes with a sequenced biological effects on the cells which may result in tumor formation (Al-Sabti, 1986). The spontaneous human melanomas can occur as a result of increased chromosomal dosage in the cells (Gabriele *et al.*, 1990, Douglas and Valerie, 1992 and Poetsche *et al.*, 1998). Thus, the spontaneous melanoma that was observed in our study can be attributed to a gene or genes amplification events reflected in the cytogenetical study as an increased chromosomal number. This observation agrees with that of Griffin *et al.* (1988).

The DNA fingerprinting analysis revealed that only two primers (OPA-05) and (OPA-09) gave positive results. A comparison between control fish and melanoma bearing fish, using the first positive primer (OPA-05) indicated the appearance of three new bands in the melanoma bearing fish, whereas the second positive primer (OPA-09) produced divergence in melanoma bearing fish than in

control. The new bands indicated the presence of additional sequence(s) in the melanomatous individuals, which could be explained as a result of gene(s) alterations responsible for melanoma formation. Adam *et al.* (1993) who attributed fish melanoma formation to a dominant genetic locus *TU* gave this suggestion. This locus includes the *Xmrk* oncogene which was known to encode a receptor tyrosine kinase. On the other hand, Schartl (1990) detected an additional copy of the *Xmrk* gene in fish which showed melanomas and attributed such cases to a gene duplication event . They also found that the spontaneous melanoma formation in *Xiphophorus* platyfish swordtail was induced not only by the six - linked oncogene *Xmrk,* but also by the autosomal tumor suppressor gene *DiFF*. Nairn *et al.* (1996a) succeeded in recovering the CDKN2-like DNA sequence by PCR amplification from *X. maculatus* genome. They found that this sequence is mapped on the *Xiphophorus* linkage group V, in the close vicinity of the *DiFF* locus.

Concerning the cytogenetic changes of experimentally gamma-irradiated fish, our results revealed that the chromosomal aberrations seem to be dose-dependent. The fish group that was exposed to a 2 Gy dose showed structural and numerical aberrations lower than that exposed to 6 Gy gamma-rays, while the effects of UV light were equivalent to that of a 2 Gy gamma- ray dose. However, the exposure to gamma-rays (2 Gy) and ultraviolet light induced chromosome gaps and centromeric attenuation, which may be attributed to the effect of radiation on DNA. On the other hand, exposure of the fish to radiation induced segregation of the *DiFF* gene, which played a role in melanoma formation (Nairn *et al.*, 1996b). In the fish group that was exposed to 4 Gy gamma rays, fragments, breaks and rings were additionally noticed as structural aberrations together with monosomics and nullisomics as numerical types of aberrations. This deleterious effect could be attributed to the effect of radiation on the DNA through the formation of pyrimidine dimers which may result in the formation of large numbers of fragments (Graham, 1993).

From our results, the mechanisms of melanoma formation are thought to be different in spontaneous cases than in that of those experimentally induced by radiation. In spontaneous cases, the tumor was produced as a result of duplication of gene(s) which may be oncogenic. The gene duplication was manifested cytogenetically in the form of trisomics and polyploidy. In case of radiation-induced melanomas, loss of specific gene(s) such as tumor supressor genes is suspected to be incriminated in melanoma induction. This loss appeared cytogenetically as fragments, gaps or deletions.

The spontaneous melanomas as well as the exposure of the fish to UV light induced an insignificant increase of micronuclei formation, while the exposure to gamma-rays with different doses induced a highly significant increase. The micronuclei frequency increased in case of pollution (Al-Sabti , 1986, Ueda *et al.*, 1992 and Das and Nanda , 1986) as well as in the case of exposure to ionizing radiations (Countryman and Heddle , 1976). The explanation of our findings is based on the biphasic action of the ionizing radiation either causing DNA damage or DNA fragmentation (Jones *et al.*, 1987). The micronuclei formation depends on broken chromosome or chromatid fragments during mitosis (Brusick , 1980).

Data obtained by the PCR technique showed that none of the primers used (even those, which gave positive results with melanoma bearing fish) produced any fingerprints in the irradiated fish samples. In DNA of fish exposed to ionizing radiations, much damage can be distinguished, single

strand breaks and double strand breaks, cross-links or nucleotide damage in base or sugar moiety. This damage may be attributed to the loss of some or all sequences needed for the tested primers. Schulte-Frohlinde (1987) and Bien *et al.* (1988) who reported that the DNA damage produced by irradiation inhibits the DNA activity by different contribution levels previously explained this observation. The inhibition of DNA activity by irradiation is due to the loss of some DNA sequences (DNA deletions) and in turn, the loss of the function of the gene(s) responsible for that sequence (Van Touw *et al.*, 1985 and Zahran , 1989).

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الملخص العربي

دراسات على التغيرات الوراثية والجزيئية للسرطانات الصبغية في أسماك الزينة نتيجة التهجين

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أجريت هذه الدراسة لإلقاء الضوء على أهم الأورام شيوعا في الأسماك وهى الأورام الصبغية. لوحظ في هذه الدراسة ظهور الأورام في عدد ٢٠ سمكة من الأسماك ذات الذيل السيفى من مجموع ٥٣٠ سمكة تم فحصها. ولقد درست هذه الأورام التلقائية بمقارنتها بمجموعة أسماك ضابطة و بمجموعة أسماك أخرى تم استحداث الورم بها تجريبيا وذلك باستخدام أشعة جاما (٢، ٤، ٦ جراى) أو الأشعة فوق البنفسجية. ولقد أظهرت الدراسة الوراثية للأسماك ذات الأورام التلقائية وجود تشوهات كروموسومية سواء عددية أو تركيبية وكذلك وجود نوى صغير بجانب النواة الأصلية لخلايا الدم الحمر خاصة في الأسماك المعرضة للإشعاع. أما بالنسبة للدراسة الجزيئية باستخدام تفاعل البلمرة الإنزيمي المتسلسل وبالاستعانة بعشرة بادئات عشوائية فلقد ظهرت فروق واضحة في الـ د ا ن للأسماك الضابطة وذات الأورام التلقائية وذلك مع بادئين فقط. أما الأسماك المعرضة للإشعاع فلم تظهر آي نتائج ايجابيه وقد يعزى ذلك لتأثير الإشعاع المدمر على الحمض النووي للأسماك. ويراعي مستقبلاً استخدام الأسماك ككاشف بيولوجي عند حدوث التلوث بالإشعاع خاصة بعد ازدياد فقدان طبقة الأوزون نتيجة النشاط الصناعي البشري ويمكن ذلك عن طريق الفحص الباثولوجي - الكروموسومي وفحص الحمض النووي (الـ د ن أ) .