ras gene mutations are a rare event in human uveal and cutaneous melanomas

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Abstract. Melanomas are malignant tumours with high metastatic potential. The genetic alterations which lead to the transformation and progression of melanocytes to malignant melanoma remain obscure. Mutations in the *ras* gene family have been described, however their role in melanoma pathogenesis is still controversial. In this study we examined the incidence of H-, K- and N-*ras* mutations in 47 DNA samples isolated from paraffin-embedded 25 cutaneous and 22 uveal malignant melanoma tissues and a MeWo melanoma cell line using the Restriction Fragment Length Polymorphism (RFLP) analysis of PCR products. Only one mutation in codon 61 of the N-*ras* gene was found suggesting that the importance of *ras* mutations in melanoma tumourigenesis may be limited.

Introduction

Carcinogenesis is considered to be a complex multistep process (1,2). Evidence from epidemiological studies in man and biochemical and histopathological observations of tumours *in vitro* and *in vivo* suggest that chemicals, radiation and viruses are involved in the initiation of the malignancy. These factors could alter, directly or indirectly, the expression of specific genes, namely oncogenes and onco-suppressor genes and affect the machinery regulating normal growth, differentiation and programmed cell death, leading to malignant transformation (2).

The *ras* gene family is frequently found to be deregulated in tumours. It consists of many related genes which share the characteristic of signal transduction. Among them, the H-*ras*, K-*ras* and N-*ras* are the most well-characterized and studied (3). The three genes encode for highly homologous proteins (p21 *ras*) which are localized at the inner side of the cell membrane and present GTPase activity.

Genetic defects characterizing the *ras* genes include amplification and loss of a normal *ras* allele, but the commonest alterations involve point mutations in codons 12, 13 and 61 of the H-, K-, and N-*ras* genes.

The selective growth advantage confered by *ras* gene mutations has been demonstrated in cell systems *in vitro* and mouse models. Chemical carcinogens have been shown to induce skin papillomas in mice with high percentage of activated H-*ras* (4) while transfection of early passage rodent cells with the mutant H-*ras* leads to malignant conversion by causing multiple metastasis when injected in nude mice (5,6). Furthemore, mutations in the *ras* genes have been described in a large range of human tumours (7,8).

Melanomas are malignant tumours with high metastatic potential. Both intrinsic and extrinsic risk factors have been discussed and studied as a possible etiology for the initiation and development of this type of tumour (9). Genetic factors may play a role, since differential incidence of melanomas has been found in different ethnic groups and family history can be a risk parameter. Furthermore, extrinsic factors, such as ultraviolet (UV) radiation, are considered as major causes of melanomas (10).

The role of *ras* oncogenes in the pathogenesis of malignant melanomas remains paradoxical (11,12). The incidence of *ras* mutations in particular is a subject of debate due to controversial data that emphasizes the confusing nature of *ras* gene alterations and the melanoma tumourigenesis.

In order to examine this question we studied the incidence of H- and K-*ras* mutations in codon 12 and of the N-*ras* mutations in codon 61 using the Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP) analysis in 47 melanomas and a MeWo human melanoma cell line.

Materials and methods

Clinical specimens and cell lines. The 22 paraffin uveal melanoma blocks were obtained from the Institute for Cancer

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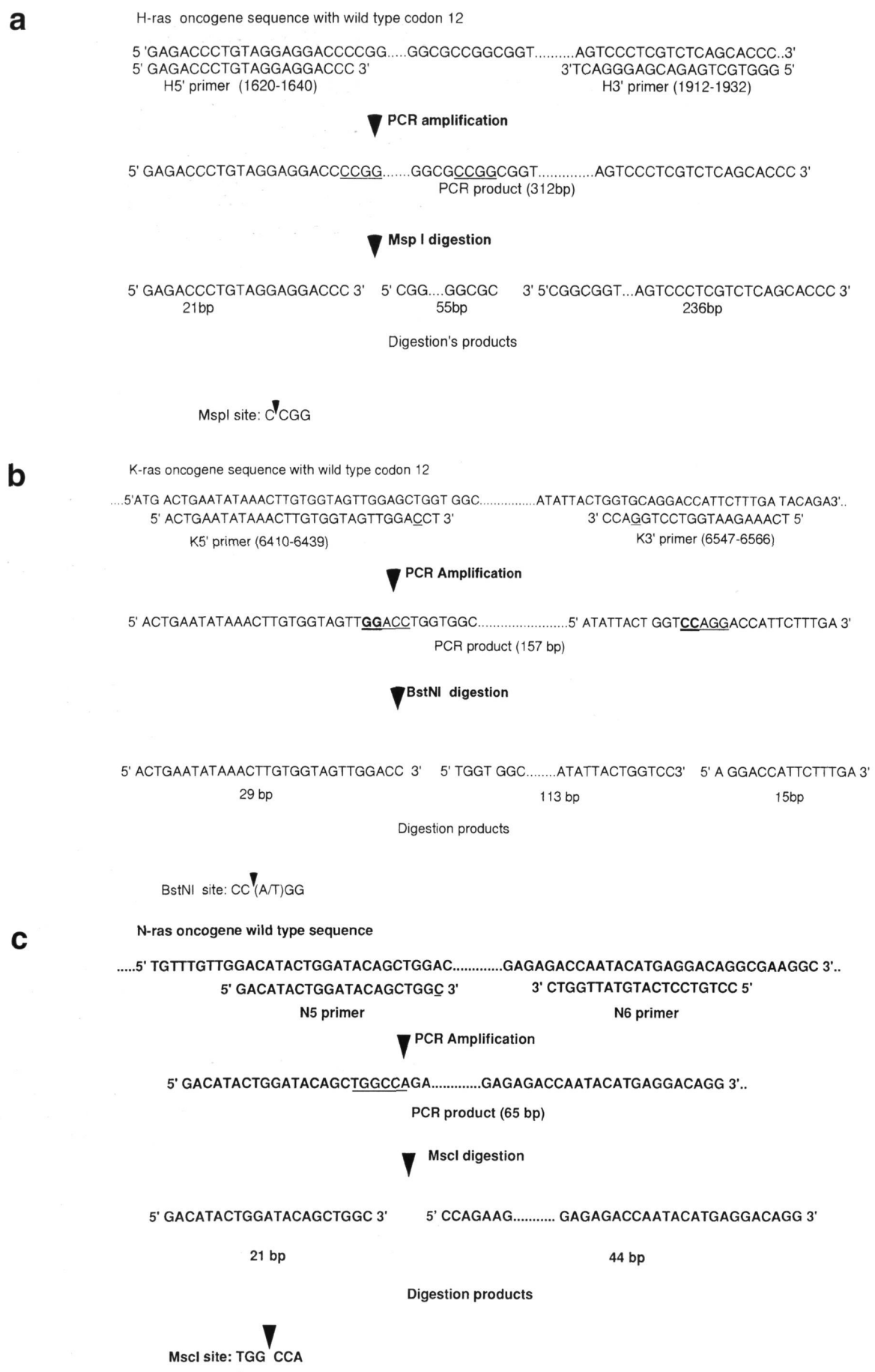

Figure 1. Schematic illustration of the detection of (a) H-*ras* codon 12, (b) K-*ras* codon 12 and (c) N-*ras* codon 61 mutations by PCR-RFLP analysis.

Studies, Sheffield, UK and the 25 cutaneous from the Medical School, University of Thrace, Alexandroupolis, Greece. Posterior uveal melanoma tissue was obtained following enucleation, fixed and embedded in paraffin wax, and processed for routine histological assessment. The samples included in this study were from ciliary and choroidal malignant melanomas.

The MeWo cell line (13) as well as the EJ human bladder carcinoma, SW480 human colon cancer and the HL60 human myeloid leukemic cell lines were maintained in RMPI-10% FCS supplemented with 2 mM glutamine.

DNA extraction. The paraffin-embedded tissues were put in 1.5 ml tubes and 300 µl lysis solution containing 0.1 N NaOH/ 2M NaCl were added. Samples were then boiled for 5 min and centrifuged at 13000 rpm for 5 min at 4°C. Supernatants were transferred into fresh tubes, extracted once with phenol/chloroform and once with chloroform alone and DNA was precipitated with the addition of 1 ml ethanol. DNA was recovered with centrifugation at 13000 rpm for 15 min at 4°C washed with cold 70% ethanol and resuspended in 20 µl distilled water. DNAs from the cell lines were isolated in a similar manner.

Oligonucleotide primers and PCR amplification. The oligonucleotides for H-*ras* codon 12, K-*ras* codon 12 and N-*ras* codon 13 shown in Fig. 1 have been previously described (14,15). 1-2 µl of the extracted DNA of each sample was amplified in a volume of 100 µl under the following conditions:

H-*ras* amplification: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 200 µM of each dNTP, 500 ng of each primer and 2.5 U *Taq* polymerase (Perkin Elmer Cetus). Amplification parameters were the following: Denaturation at 94°C for 1 min, primer annealing and extention at 68°C for 1 min, 35 cycles.

K-*ras* amplification: 16.6 mM $(NH4)_2SO_4$, 10 mM ß-mercaptoethanol, 6.7 mM $MgCl_2$, 67 mM Tris-HCl pH 8.8, 1.7 mg BSA, 200 µM of each dNTP, 500 ng of each primer and 2.5 U *Taq* polymerase (Perkin Elmer Cetus). Amplification parameters were the following: Denaturation at 94°C for 40 sec, primer annealing at 60°C for 45 sec and extention at 72°C for 50 sec, increasing the extention time by 1 sec per cycle, 40 cycles.

N-*ras* amplification: 16.6 mM $(NH4)_2SO_4$, 10 mM, ß-mercaptoethanol, 6.7 mM $MgCl_2$, 67 mM Tris-HCl pH 8.8, 1.7 mg BSA, 200 µM of each dNTP, 500 ng of each primer and 2.5 U *Taq* polymerase (Perkin Elmer Cetus). Amplification parameters were the following: Denaturation at 94°C for 40 sec, primer annealing at 55°C for 50 sec and extention at 72°C for 50 sec, 40 cycles.

RFLP analysis. H-*ras* codon 12: 10-20 µl aliquots of the amplification products were digested O/N with 20 U MspI.

K-*ras* codon 12: 10-20 µl aliquots of the amplification products were digested for 3 h with 20 U BstNI.

N-*ras* codon 61: 10-20 µl aliquots of the amplification products were digested O/N with 20 U MscI.

Enzymes were purchased from New England Biolabs and the conditions followed for digestions were those recommended by the supplier. Incubation temperatures were 60°C for BstNI and 37°C for MspI and MscI.

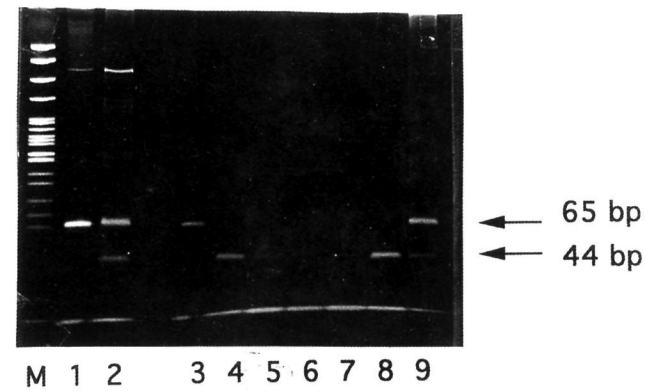

Figure 2. N-*ras* amplification products digested with MscI and electrophoresed through an 8% polyacrylamide gel. Lane 1: Undigested positive control (HL60 cell line) (65 bp), Lane 2: Positive control digested with MscI, Lane 3: undigested PCR product, Lanes 4-8: Negative samples (44 bp), Lane 9: Positive sample, M: pBR322/MspI DNA marker. Resistance to MscI digestion suggests a mutated N-*ras* gene (codon 61).

The EJ and the SW480 cells have mutant H- and K-*ras* alleles in codon 12 and the HL60 cell line has a mutated N-*ras* codon 61 allele. PCR products from amplified DNA isolated from these cell lines were used as positive controls for the completion of the digestion with MspI, BstNI and MscI, respectively.

Digestion products were electrophoresed through a 2% agarose gel (for H- and K-*ras* products) or an 8% native polyacryamide gel (for N-*ras* products). Gels were stained with ethidium bromide and photographed on a UV light transilluminator.

Results and Discussion

The role of oncogenes in melanoma tumourigenesis is still controversial. The incidence of *ras* mutations, in particular, is a subject of debate. Initial studies showed that approximately 20% of cutaneous melanomas presented *ras* mutations, the majority of which was found in the N-*ras* gene (10,16). Furthermore, these mutations occured in or adjacent to dipyrimidine sites, which is a well known target for UV damage (10). Albino *et al* (17) found similar results in cultured melanoma cell lines although only 5-6% of non-cultured primary and metastatic melanomas had mutated *ras* genes. Again, the N-*ras* codon 61 mutations were found to predominate. However, some reports argue the importance of N-*ras* in melanoma tumourigenesis. Mooy *et al* (18), failed to observe any N-*ras* mutations among the 29 DNA samples from uveal melanoma tested with asymmetric PCR and sequencing. Similarly, O'Mara *et al* (19) by studying 22 melanoma short-term cell lines for N-*ras* mutations, identified only two, both found in codon 61 of the gene. Limited information is available on the molecular genetics of uveal melanomas. Increased *myc* expression has been reported and loss of heterozygosity of THRB and *nm*23-H1 gene have been found (20,21).

In our study, by using a PCR-RFLP (Restriction Fragment Length Polymorphism) method in 47 DNA

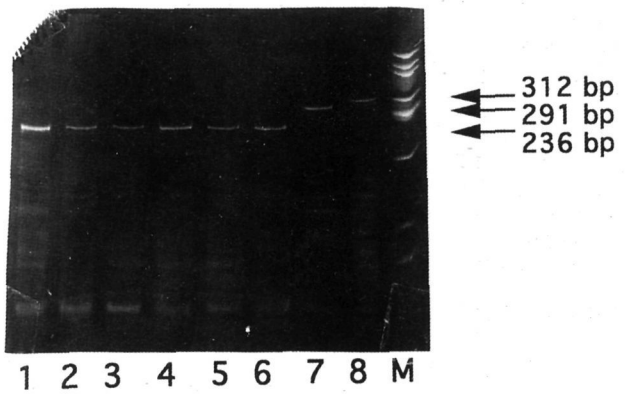

Figure 3. H-*ras* amplification products digested with MspI and electrophoresed through a 2% agarose gel. Lanes 1-6: Negative samples (236 bp), Lane 7: Positive control (EJ cell line) (291 bp), Lane 8: undigested product (312 bp), M: pUC18/HaeIII DNA marker.

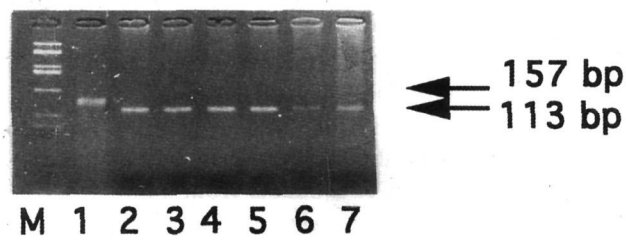

Figure 4. K-*ras* amplification products digested with BstNI and electrophoresed through a 2% agarose gel. Lane 1: Positive control (SW480 cell line) (157 bp), Lanes 2-7: Negative samples (113 bp), M: pUC18/HaeIII DNA marker.

samples isolated from paraffin-embedded sections of 22 uveal and 25 cutaneous melanomas, we detected only one N-*ras* codon 61 mutation (Fig. 2, lane 9) in a uveal melanoma. Wild type product (44 bp) was identified in this sample, indicating heterozygosity for the mutation, which is in agreement with a previous report (19). No N-*ras* mutations were found in the 25 cutaneous melanomas tested suggesting that the incidence of N-*ras* mutations in this type of malignancy has been possibly overestimated, and similar results are shown for uveal melanomas. Uveal melanomas show very few cytogenetic abnormalities, compared with other solid tumours, and relatively little stromal tissue and cellular infiltration (22,23). The results of our study would therefore preclude a role for N-*ras* in the early events of tumourigenesis of melanoma.

Furthermore, we found no H- and K-*ras* codon 12 mutations (Figs. 3 and 4), which is in agreement with some but not all previous reports on cutaneous melanomas. A high percentage of K-*ras* mutations was reported by Shukla *et al* (24) in 40 melanotic lesions, using oligodeoxynucleotide hybridization of PCR products. On the contrary, Albino *et al* (25), by following similar experimental procedure, found no mutations at codon 12 in the K-*ras* gene. We also failed to observe any *ras* mutation in the melanoma cell line MeWo.

The heterogeneity of factors which could contribute to the initiation and/or the development of melanomas could partly explain the marked discrepancy in the mutation frequencies observed. Although both our study and those of others suggest that mutations in *ras* genes are rare in melanomas, a possible role for *ras* for the development and/or progression of this type of malignancy should not be excluded. Long- but not short-term expression of the viral H-*ras* gene has been shown to induce a complete transformed phenotype in melanocytes, possibly via introduction of chromosomal instability (26). Additional factors such as paracrine and autocrine functions and their possible interaction with the *ras*-mediated signal transduction may also be important. Indeed, melanoma cells produce a variety of growth factors and growth factor receptors and fibroblast growth factor (FGF) appears to have an autocrine role (27). Since *ras* has been shown to mediate the signal of several growth factors, including that of FGF (28), the levels of the p21 *ras* protein could be important. Increased levels of p21 *ras* protein in melanoma cells, as compared to normal melanocytes were described by Tanikawa *et al* (29) although Albino *et al* (17) found no difference in *ras* p21 expression between melanomas at different phases of growth or between those harboring a known *ras* mutation and those that did not. Finally, it has been suggested that mutated *ras* genes are more interactive with the machinery controlling melanocyte differentiation rather than transformation and *ras* gene mutations have been correlated with a subset of EGF receptor and Class II major histocompatibility (Ia) antigen positive melanomas from an early phase of differentiation (17).

The complexity of the up-to-date information clearly suggest that a persistent and cautious approach must be followed in order to unravel the precise role of *ras* gene family in melanoma tumourigenesis.

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