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ORIGINAL PAPER

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Analysis of *ras* mutations in human melanocytic lesions: activation of the *ras* gene seems to be associated with the nodular type of human malignant melanoma

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Abstract We have analyzed the Ha-*ras*, Ki-*ras* and N-*ras* gene for point mutations at codons 12, 13 and 61 via restriction fragment length polymorphism/polymerase chain reaction analysis and subsequent direct sequencing in non-cultured fresh-frozen tissues of 16 superficial spreading melanomas (SSM), 13 nodular malignant melanomas (NMM), 2 lentigo malignant melanomas (LMM), 1 dysplastic nevus, 1 congenital nevus and 5 normal nevi from 38 patients. Mutations were found in 4 melanoma samples, all belonging to the nodular malignant type. Three of them were mutated in N-*ras* and one in the Ha-*ras* gene. Mutation in N-*ras* was also detected in the congenital nevus. All mutations were exclusively located at the first two base pairs of codon 61. No Ki-*ras* mutation was detected in any lesion. No mutation could be found in SSM and LMM in addition to dysplastic and normal nevi. The frequency of *ras* mutation in NMM was 31%, whereas in SSM it was 0%. Our study suggests (a) an association between *ras* mutations (mainly N-*ras*) and the NMM as a subgroup of human melanoma; (b) that activation of Ki-*ras* is not involved in the pathogenesis of melanoma. The role of UV radiation in point mutations of *ras* genes in human melanoma is discussed.

Key words Melanoma · Ha-*ras* · Ki-*ras* · N-*ras*
Oncogene · UV

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Abbreviations *PCR* polymerase chain reaction, *RFLP* restriction fragment length polymorphism, *UVR* ultraviolet radiation, *NMM* nodular malignant melanoma, *LMM* lentigo malignant melanoma, *SSM* superficial spreading melanoma

Introduction

The evolution of malignant melanoma is thought to be a multi-step process, in which a series of genetic and epigenetic events lead to the emergence of a cell clone that has escaped the normal growth control mechanisms. Principal candidates involved in these events are the members of the *ras* oncogene family, which all encode 21-kDa GTPase proteins (p21), which are related structurally, functionally and immunologically (Bos 1988). Mutation of Ha-, Ki-, and N-*ras* genes in human melanoma has previously been reported and evidence has accumulated, that the N-*ras* gene, preferentially mutated at the 61st codon, is the predominant *ras* gene activated in malignant melanoma. The exact role of the *ras* oncogene in the pathogenesis of malignant melanoma remains unclear, particularly because of extreme variations published mutation frequencies (Raybaud et al. 1988; Albino et al. 1989; van't Veer et al. 1989; Schrier et al. 1991; O'Mara et al. 1992). Concerning the participation of the Ki-*ras* gene in human melanoma, published results are conflicting (Shukla et al. 1989; Albino et al. 1991). We have analyzed the Ha-*ras* (Sekiya et al. 1984), Ki-*ras* (Mc Grath et al. 1983) and N-*ras* genes (Hall and Brown 1985) at codons 12, 13 and 61 for point mutations by restriction fragment length polymorphism/polymerase chain reaction (RFLP-PCR) (Todd et al. 1991) and subsequent sequencing. This method represents a rapid and reliable alternative to the commonly used PCR-based oligodeoxynucleotide hybridization technique. Its high level of sensitivity has been further increased in our laboratory by a technical modification (Papp et al. 1993).

In order to prevent inconsistent results because of possible secondary genetic alterations in cultured cell

lines, we exclusively used fresh and snap-frozen tumor samples derived from surgical resections.

In summary our results are considered under the following headings:

1. Frequency of *ras* mutations in melanoma and normal tissue
2. Comparison of *ras* mutation frequencies in Ha-*ras*, Ki-*ras* and N-*ras* genes
3. Association of *ras* mutations to a specific subset of melanoma
4. Association of *ras* mutations with extent of sun exposure.

According to the enormous heterogeneity in the biological features of human melanoma, the identification of specific melanoma subtypes by PCR screening, could help to generate more efficient diagnosis and therapy strategies in particular cases.

Materials and methods

Tissue samples

Snap-frozen tumor samples of 18 human primary malignant melanomas (14 superficial spreading melanoma, SSM, and 4 nodular malignant melanoma, NMM), 13 metastases (originating from 2 SSM, 9 NMM and 2 lentigo malignant melanoma, LMM), 1 dysplastic nevus, 1 congenital nevus and 5 normal nevi from 38 patients originating from surgical resections obtained from the Dermatology Department of the University Hospital of Würzburg.

DNA preparation

Tumor samples were crushed to powder under liquid nitrogen by a sterile pestle and mortar. High-molecular-mass genomic DNA was extracted by incubating the minced tissue for 20 h at 50 °C in 2 ml TE buffer (10 mM TRIS, pH 8, 0.1 mM EDTA) containing 100 µl 20% sodium dodecyl sulfate (SDS) and 100 µl of proteinase K solution (10 mg/ml), followed by two phenol/chloroform extractions and a subsequent chloroform extraction (Davies et al. 1986). The volume was reduced to 200 µl under vacuum in a Speedvac Concentrator (Uniequip). Finally, the DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 6) and 2.5 volumes of 100% ethanol, washed with 70% ethanol and resuspended in an appropriate volume of sterile, deionized and distilled water.

Electroelution

In order to remove PCR-inhibiting elements, 10 µg genomic tumor DNA was run on a 1% agarose gel in 1 × TAE buffer (40 mM TRIS, pH 7.2, 20 mM acetic acid, 1 mM EDTA) for 2 h at 80 mA. High-molecular-mass DNA was excised and electroeluted (HSB Elutor, Biometra) with 1 × TAE buffer for 1 h at 100 V and trapped in 70 µl 3 M sodium acetate (pH 6). Salts were removed by washing three times with 1.5 ml sterile, deionized and distilled water in Centricon 30 Concentrators (Amicon) at 5000 *g* in a 34 ° angle rotor. The purified DNA (40 µl) was stored at −20 °C.

General PCR conditions

All PCRs were performed in a 100 µl volume according to standardized basic conditions (Erlich 1989) containing 50 mM KCl, 10 mM TRIS/HCl (pH 8.4 at room temperature), 1.5 mM $MgCl_2$, 100 µg/µl gelatin, 200 µM each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP) and 2.5 units Taq polymerase, using varying amounts of template and primers. Samples were denatured for 3 min at 95 °C, incubated for 35 cycles (one cycle: 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C) and finally extended for 7 min at 72 °C. Amplification was performed using a DNA thermal cycler (Perkin Elmer).

All primers were synthesized by the oligonucleotide synthesizer Gene Assembler Plus under conditions recommended by the supplier (Pharmacia).

RFLP-PCR primers

The nucleotide sequence of all primers in conjunction with the respective screening strategy are described in Table 1 (N-*ras*, Ki-*ras*) and Table 2 (Ha-*ras*).

Preamplification

In order to increase the amount of material available for examination, 1 µl electroeluted and purified genomic tumor DNA was preamplified with 300 ng amplification primers for exons 1 and 2 of the N-*ras* and Ki-*ras* genes respectively (Table 1). For the Ha-*ras* gene we have recently described endogenous restriction sites that can detect all possible activating point mutations without the necessity of using mismatch primers (Papp et al. 1992) (Table 2). Sense oligo-H5′12 and antisense oligo-H3′61 were used for simultaneous amplification of exons 1 and 2. In this case the resulting 555-base-pair (bp) fragment was used as the preamplification product. 10% of the preamplification product along with positive and negative controls and the 123-bp ladder, as a marker, were evaluated by gel electrophoresis on a 1% agarose gel and ethidium bromide fluorescence visualization. Finally, 1–5 µl preamplification product was diluted with 1 ml sterile, deionized and distilled water and stored at −20 °C.

RFLP-PCR

A 10 µl sample of the diluted preamplification product was amplified with 150 ng mismatch primer (Ki-*ras*, N-*ras*) or 5′primer (Ha-*ras*) and 150 ng 3′primer; 10% of the amplification product was evaluated by agarose gel electrophoresis, prior to subsequent Centricon 30 purification of the remaining product. An appropriate aliquot was digested with 10 units restriction endonuclease and resolved by gel electrophoresis on a 3.5% NuSieve 1% agarose gel. A mutation is visualized as a persistent undigested fragment after restriction enzyme cleavage.

The RFLP-PCR method has an approximate level of sensitivity of 5% mutant sequences (O'Mara et al. 1992).

Asymmetric PCR and direct sequencing

In order to exclude the possibility of false positive screening signals and to characterize the exact mutation specificity, it is generally necessary to confirm all mutations by subsequent sequencing of the corresponding PCR product.

The accurate sequence of a mutated allele can be rapidly determined by direct sequencing of the asymmetrically amplified PCR product. A mutation is demonstrated as an additional band in the sequencing ladder. A single-stranded sequencing template was generated by performing a PCR with 10% purified RFLP-PCR product and 900 ng mismatch primer. The sequencing template was purified by electroelution; 25% of the electroeluted single strand was sequenced by means of 30 ng sequencing primer (antisense oligonucleotide), with the Sequenase 2.0 kit (USB) and [α-^{35}S]dATP (Amersham) in accordance with the recommendations of the suppliers.

Modified RFLP-PCR (Papp et al. 1993)

Some RFLP-PCR products resulted in digestions with extremely faint residual fragments. Consequently the corresponding mutation could not be identified as a distinct additional band in the sequencing ladder by

Table 1 Restriction fragment length polymorphism/polymerase chain reaction (RFLP-PCR) assay for the N-*ras* and Ki-*ras* gene

Primers N-*ras* Codons 12, 13 and 61		Primers Ki-*ras* Codons 12, 13 and 61	
Exon 1		Exon 1	
Amplification primers:		Amplification primers:	
(+ strand)	(– strand)	(+ strand)	(– strand)
N5′12 CTGGT′GTGAA′ATGAC′TGAGT	N3′12 GGTGG′GATCA′TATTC′ATCTA	K5′12 CCTGC′TGAAA′ATGAC′TGAAT	K3′12 TGTTG′GATCA′TATTC′GTCCA
Mismatch primers:		Mismatch primers:	
(+ strand)		(+ strand)	
N12m AACTG′GTGGT′GGTTG′GAGCC	(*Msp*I C!CGG codon 12, nucleotides 1, 2)	K12m TAAAC′TTGTG′GTAGT′TGGAG′CC	(*Msp*I C!CGG codon 12, nucleotides 1, 2)
digested PCR product: 19 bp and 73 bp		digested PCR product: 21 bp and 73 bp	
(+ strand)		(+ strand)	
N13m3 CTGGT′GGTGG′TTCCA′GCAGG′T	(*Pfl*MI CCANNNN!NTGG codon 13, nucleotides 1, 2)	K13m ACTTG′TGGTA′GTTCC′AGCTG′GT	(*Pfl*MI CCANNNN!NTGG codon 13, nucleotides 1, 2)
digested PCR product: 19 bp and 71 bp		digested PCR product: 20 bp and 71 bp	
Sequencing primer:		Sequencing primer:	
(– strand)		(– strand)	
SN12 TTCAT′CTACA′AAGTG′GT		SK12 TTCGT′CCACA′AAATG′ATTC	
Exon 2		Exon 2	
Amplification primers:		Amplification primers:	
(+ strand)	(– strand)	(+ strand)	(– strand)
N5′61 GTTAT′AGATG′GTGAA′ACCTG	N3′61 ATACA′CAGAG′GAAGC′CTTCG	K5′61 GTAAT′TGATG′GAGAA′ACCTG	K3′61 ATACA′CAAAG′AAAGC′CCTCC
Mismatch primers:		Mismatch primers:	
(+ strand)		(+ strand)	
N61mA GACAT′ACTGG′ATACA′GCTGG′C	(*Msc*I TGG!CCA codon 61, nucleotides 1, 2)	K61mA GATAT′TCTCG′ACACA′GCAGG′C	(*Hae*III GG!CC codon 61, nucleotide 1)
digested PCR product: 20 bp and 65 bp		digested PCR product: 20 bp and 65 bp	
(+ strand)		(+ strand)	
N61mB2 GGACA′TACTG′GATAC′AGCTG′GTCT	(*Xba*I T!CTAGA codon 61, nucleotide 3)	K61mB ATATT′CTCGA′CACAG′CAGGT′T	(*Mse*I T!TAA codon 61, nucleotides 2, 3)
digested PCR product: 22 bp and 64 bp		digested PCR product: 20 bp and 64 bp	
Sequencing primer:		Sequencing primer:	
(– strand)		(– strand)	
SN61 AGCCT′TCGCC′TGTCC′TC		SK61 GAAAG′CCCTC′CCCAG′TC	

```
               +-----------CC-N13m3>
---N5'12->   +--------------N12m-C
ATGACTGAGTACAAACTGGTGGTGGTTGGAGCAGGTGGTGTTGCGAAAAG    50
---------+---------+---------+---------+---------+
TACTGACTCATGTTTGACCACCACCAACCTCGTCCACCACAACGCTTTTC
                                 #12#13
                                 GlyGly

CGCACTGACAATCCAGCTAATCCAGAACCACTTTGTAGATGAATATGATC   100
---------+---------+---------+---------+---------+
GCGTGACTGTTAGGTCGATTAGGTCTTGGTGAAACATCTACTTATACTAG
                          <-SN12----------+
                                   <-N3'12--------

   Exon 1 >< Exon 2             +-----------N5'61-
CCACCATAGAGGATTCTTACAGAAAACAAGTGGTTATAGATGGTGAAACC   150
---------+---------+---------+---------+----------
GGTGGTATCTCCTAAGAATGTCTTTTGTTCACCAATATCTACCACTTTGG

----+

        +-------------N61mB2-T-T
->       +-------------N61mA-C
TGTTTGTTGGACATACTGGATACAGCTGGACAAGAAGAGTACAGTGCCAT   200
---------+---------+---------+---------+---------+
ACAAACAACCTGTATGACCTATGTCGACCTGTTCTTCTCATGTCACGGTA
                              #61
                              Gln

GAGAGACCAATACATGAGGACAGGCGAAGGCTTCCTCTGTGTATTTGCCA   250
---------+---------+---------+---------+---------+
CTCTCTGGTTATGTACTCCTGTCCGCTTCCGAAGGAGACACATAAACGGT
               <-SN61----------+
                        <-N3'61------------+
```

```
              +------------CC-K13m->
---K5'12-> +---------------K12m-C
ATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAG    50
---------+---------+---------+---------+---------+
TACTGACTTATATTTGAACACCATCAACCTCGACCACCGCATCCGTTCTC
                                 #12#13
                                 GlyGly

TGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATC   100
---------+---------+---------+---------+---------+
ACGGAACTGCTATGTCGATTAAGTCTTAGTAAAACACCTGCTTATACTAG
                        <-SK12------------+
                                   <-K3'12--------

   Exon 1 >< Exon 2             +-----------K5'61-
CAACAATAGAGGATTCCTACAGGAAGCAAGTAGTAATTGATGGAGAAACC   150
---------+---------+---------+---------+---------+
GTTGTTATCTCCTAAGGATGTCCTTCGTTCATCATTAACTACCTCTTTGG

----+

          +-------------K61mB-T
->       +-------------K61mA-C
TGTCTCTTGGATATTCTCGACACAGCAGGTCAAGAGGAGTACAGTGCAAT   200
---------+---------+---------+---------+---------+
ACAGAGAACCTATAAGAGCTGTGTCGTCCAGTTCTCCTCATGTCACGTTA
                              #61
                              Gln

GAGGGACCAGTACATGAGGACTGGGGAGGGCTTTCTTTGTGTATTTGCCA   250
---------+---------+---------+---------+---------+
CTCCCTGGTCATGTACTCCTGACCCCTCCCGAAAGAAACACATAAACGGT
                  <--SK61---------+
                        <-K3'61------------+
```

subsequent sequencing, because this band was too weak and therefore blotted out by non-specific background signals. In these cases, the RFLP-PCR product again was digested and resolved on an agarose gel. But now the faint residual fragment was carefully excised and electroeluted. The eluate then was reamplified under identical conditions (enrichment of the mutated allele). A sample comprising 10% of the reamplification product was again evaluated by agarose gel electrophoresis, prior to subsequent Centricon 30 purification of the remaining product. Once more, an appropriate aliquot was digested and resolved by gel electrophoresis. In the case of a mutation, the resulting fragment then was almost indigestible and the sequence of the mutated allele could easily be resolved by subsequent asymmetric sequencing of the corresponding PCR product.

In order to rule out possible Taq polymerase errors, generally all screened mutations were verified by two repetitions of the respective RFLP-PCR and sequencing strategy.

T-vector cloning and sequencing

The faint residual fragment, corresponding to the mutated Ha-*ras* allele, could not be selected by the modified RFLP-PCR technique because of

Table 2 RFLP-PCR assay for the Ha-*ras* gene (Sekiya et al. 1984; Kahn et al. 1990; Papp et al. 1992)

Amplification primers and sequencing primer for exon 1 of the Ha-*ras* gene

	(+ strand)		(– strand)
H5′12	5′GAGAC CCTGT AGGAG GACCC 3′	H3′12	5′GGGTG CTGAG ACGAG GGACT 3′
			(– strand)
Sequencing primer for codons 12 and 13		SH12	5′TTCGT CCACA AAATG GT 3′

Gene and target: human Ha-*ras* codon 12 — endogenous restriction site: *Msp*I (C!C**GG**)
Detection of point mutations at first and second nucleotide of codon 12
Fragment size(s)

Undigested allele:	312 bp
Digested normal allele:	236 bp + 56 bp + 20 bp
Digested mutant allele:	292 bp + 20 bp

Gene and target: human Ha-*ras* codon 13 — endogenous restriction site: *Aci*I (G!C**GG**)
Detection of point mutations at first and second nucleotide of codon 13
Fragment size(s):

Undigested allele:	312 bp
Digested normal allele:	89 bp + 54 bp + 118 bp + 26 bp + 25 bp
Digested mutant allele:	143 bp + 118 bp + 26 bp + 25 bp

Amplification primers and sequencing primer for exon 2 of the Ha-*ras* gene

	(+ strand)		(– strand)
H5′61	5′GTCAT TGATG GGGAG ACGTG 3′	H3′61	5′ACACA CACAG GAAGC CCTCC 3′
			(– strand)
Sequencing primer for codon 61		SH61	5′TCCCC GGTGC GCATG TA 3′

Two independant restriction assays are necessary for the screening of all possible point mutations at all three nucleotides of codon 61

Gene and target: human Ha-*ras* codon 61
Endogenous restriction site: *Bst*NI (CC!W**GG**) W = A or T
Detection of point mutations at all nucleotides of codon 61 except for A to T transversion at nucleotide 2
Fragment size(s):

Undigested allele:	112 bp
Digested normal allele:	63 bp + 15 bp + 34 bp
Digested mutant allele:	78 bp + 34 bp

Gene and target: human Ha-*ras* codon 61
Endogenous restriction site: *Cfr*I (Y!GGC**CR**) Y = C or T R = G or A
Detection of point mutations at nucleotides 1 and 2 of codon 61 except for A to G transition at nucleotide 2
Fragment size(s):

Undigested allele:	112 bp
Digested normal allele:	67 bp + 45 bp
Digested mutant allele:	112 bp

an additional constitutive *Bst*NI restriction site at codon 56 (Sekiya et al. 1984). Therefore the accurate mutation specificity was determined by ligation of the undigested PCR product into a modified M13mp18 sequencing vector. Because of the proven suitability of the *Eco*RV site in the construction of T vectors with high PCR cloning efficiency (Marchuk et al. 1991; Holton and Graham 1991), we have inserted the 60-bp *Pst*I/*Kpn*I fragment of the pG+host4 vector (Appligene) with its intrinsic *Eco*RV site inversely but in-frame into the single-strand-producing M13mp18 vector (Appligene). Transfection and cloning of the engineered vector in DH5aF′IQ competent cells was performed according to the protocol enclosed with the transfection kit (BRL). By using [α-^{35}S]dATP (Amersham) and the Sequenase 2.0 kit (USB), we were able to generate high-quality sequence ladders of the mutated as well as the non-mutated allele.

Results

Frequency of point mutations

In 31 tumor samples of primary and metastatic melanomas, mutation of the *ras* gene was detected in 4 cases (13%). In addition, activation of the *ras* gene could be demonstrated in the congenital nevus.

The 5 benign nevi and the dysplastic one were free of *ras* mutations.

Involvement of the three *ras genes*

The N-*ras* gene was mutated in 4 cases, whereas the Ha-*ras* gene was affected in only 1 case. No point mutation of the Ki-*ras* gene was detectable in any melanocytic lesion.

Ras mutation and histopathological subtype of melanoma

In the malignant melanomas investigated, all mutated *ras* genes were exclusively found in the nodular subtype. We were able to demonstrate point mutations in 4 out of 13 NMM. In 18 other types of malignant melanoma (16 SSM and 2 LMM) no *ras* mutation was found.

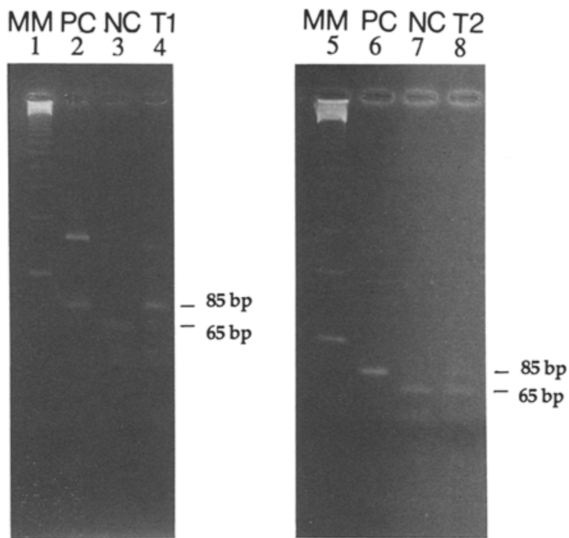

Fig. 1 Restriction fragment length polymorphism/polymerase chain reaction (RFLP-PCR) analysis of the N-*ras* gene at codon 61 by 3.5% NuSieve 1% agarose gel and ethidium bromide fluorescence visualization. Samples were amplified with the primer pair N61 mA/N3′61 and digested with *Msc*I. PCR product length = 85 bp, digested mutant allele = 85 bp, digested normal allele = 65 bp (bands above 123 bp constitute unspecific amplification products). *Lanes 1, 5* 123-bp molecular marker (*MM*); *lanes 2, 6* undigested PCR product (85 bp) (amnion DNA) as positive control (*PC*); *lanes 3, 7 Msc*I-digested PCR product (65 bp) (amnion DNA) as negative control (*NC*); *lanes 4* (*T1*), *8* (*T2*) *Msc*I-digested PCR product (85 bp and 65 bp) derived from 2 nodular malignant melanomas (NMM). The mutation of the NMM in lane 4 (T1) is clearly visualized as a distinct persistent 85-bp fragment after *Msc*I restriction enzyme cleavage and could easily be specified by subsequent direct sequencing of the corresponding PCR product. The mutation of the NMM in lane 8 (T2) is visualized as a faint persistent 85-bp fragment after *Msc*I restriction enzyme cleavage and could not be specified by direct sequencing without previous selection of the mutated allele by the modified RFLP-PCR technique (Fig. 2)

Thus the frequency of the mutation was 31% for NMM and 0% for SSM and LMM. This result suggests an association between *ras* gene activation and NMM as a histopathological subtype (χ^2: $\alpha = 2\%$)

Characterization of point mutations

In all samples with an activated *ras* gene the mutation was located at codon 61: CAA (Gln) to C**G**A (Arg) transition at codon 61 of the N-*ras* gene was found in 2 metastatic NMM (Figs. 1, 2); CAG (Gln) to C**G**G (Arg) transition at codon 61 of the Ha-*ras* gene was found in 1 metastatic NMM (Fig. 3); CAA (Gln) to **A**AA (Lys) transversion at codon 61 of the N-*ras* gene was found in 1 primary NMM as well as in the congenital nevus.

Localisation of tumor lesions

Melanomas with a mutated *ras* gene were located on the back (3 cases) and upper arm (1 case), which constitute intermittently but possibly intensely sun-exposed body sites.

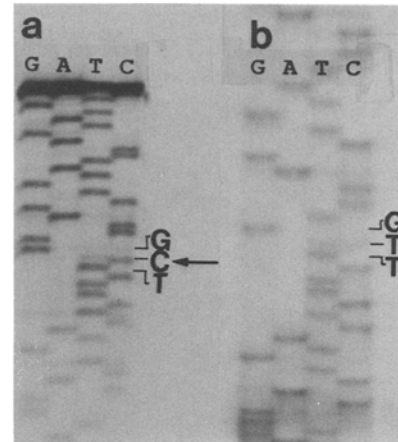

Fig. 2a, b Direct sequencing of asymmetric amplified PCR products of the same tumor sample (metastatic NMM; Fig. 1, lane 8, T2) with Sequenase 2.0, [α-^{35}S]dATP and antisense oligo SN61 as sequencing primer. **a** Modified RFLP-PCR product was asymmetrically amplified with N61 mA and sequenced with SN61; because of the selection of the mutant allele against the normal one, the TTG to T**C**G transition at the second position of codon 61, converting the amino acid Gln to Arg, is clearly visible (*arrow*). **b** The electroeluted pre-amplification product was asymmetrically amplified with N5′61 and sequenced with SN61; as the quantity of the mutant allele is too small, only the sequence of the normal allele is visible

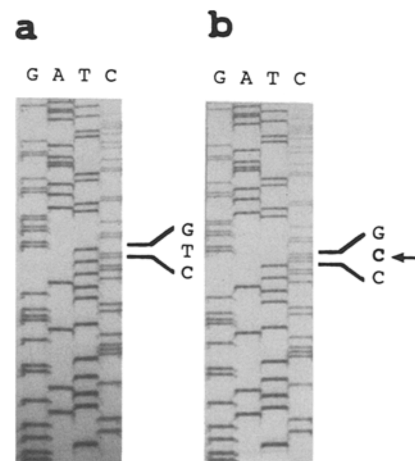

Fig. 3a, b Autoradiography of the 112-bp human HRAS1 PCR fragment of the NMM, cloned into the novel M13mp18 *Eco*RV T vector and sequenced with the (−40) primer (Sequenase 2.0 kit, USB). PCR-amplified tumor DNA was screened for point mutations in codon 61 via RFLP-PCR (Papp et al. 1992). **a** The sequence of the normal allele. **b** The CTG to C**C**G transition (*arrow*) at the second position of the antisense strand in codon 61 is shown

Discussion

The indications for a direct contribution of *ras* oncogenes to the pathogenesis of human malignant melanoma were derived from transfection studies. It was demonstrated, that the short-term expression of v-Ha-*ras* in human melanocytes drives the cells towards a transformed phenotype, with the cells gradually acquiring a subset of the character-

Table 3 Summary of *ras* mutations in human melanoma (Sekiya et al. 1984; Padua et al. 1985; Raybaud et al. 1988; Albino et al. 1989; van't Veer et al. 1989, Schrier et al. 1991; O'Mara et al. 1992; Daya-Grosjean et al. 1993; own results)

N-*ras* gene codon 61	Gln (CAA) to Lys (AAA)	12 tumors	37.5%
	Gln (CAA) to Arg (CGA)	9 tumors	28.1%
	Gln (CAA) to Leu (CTA)	1 tumor	3.1%
N-*ras* gene codon 13	Gly (GGT) to Val (GTT)	3 tumors	9.3%
	Gly (GGT) to Asp (GAT)	2 tumors	6.2%
	Gly (GGT) to Arg (CGT)	1 tumor	3.1%
N-*ras* gene codon 12	Gly (GGT) to Asp (GAT)	1 tumor	3.1%
H-*ras* gene codon 61	Gln (CAG) to Lys (AAG)	1 tumor	3.1%
	Gln (CAG) to Leu (CTG)	1 tumor	3.1%
	Gln (CAG) to Arg (CGG)	1 tumor	3.1%

istic features of melanomas (Albino et al. 1986). Furthermore, activation of Ha-*ras* and particularly N-*ras* protooncogenes by point mutations, in primary, metastatic and cultured melanomas, has been reported by several authors (Sekiya et al. 1984; Padua et al. 1985; Raybaud et al. 1988; Albino et al. 1989; Keijzer et al. 1989; van't Veer et al. 1989; Schrier et al. 1991; O'Mara et al. 1992).

Despite these observations, the exact role of *ras* oncogenes in melanoma formation remains unclear. There are considerable discrepancies in published mutation frequencies and generally only a minor proportion of the investigated tumors exhibit mutated *ras* oncogenes. Comparing cell lines with and without point-mutated *ras* genes by means of flow cytometry analysis, Albino et al. (1989) found no differences in p21 expression. Consequently, a larger number of human melanomas seem to be totally unmodified in the *ras* proto-oncogenes, in sequence as well as in expression.

We found no *ras* mutations in 5 benign nevi and the dysplastic one, as possible precursor lesions. This is consistent with results of Albino et al. (1989) and Raybaud et al. (1988), suggesting that *ras* mutations may be a late event in the development of melanoma.

One possible explanation for the facts mentioned above is that the activation of *ras* is associated with a particular subgroup of melanoma, characterized by (a) a certain histopathological type, (b) a specific differentiation stage (c) a specific etiological agent (e.g. UV radiation), (d) other genetic alterations, or a combination of these.

Some previously published investigations together with our findings support this idea:

1. Our results suggest that there may be an association between the mutation of the *ras* gene and the nodular malignant melanoma (NMM) as a histopathological subtype. In contrast, we could not detect any *ras* mutations in the superficial spreading and lentigo malignant melanomas (SSM and LMM) (total $\chi^2 = 5.878$, $\alpha < 2\%$) nor has this been reported previously.

Thus, the high variability of reported mutation frequencies in melanomas (5%, 8%, 9%, 13%, 24%, 27%) (Albino et al. 1989; Raybaud et al. 1988; O'Mara et al. 1992; our own results; van't Veer et al. 1989; Schrier et al. 1991) may be explained by differences in the relative amounts of NMM and SSM in the tumor samples investigated. Furthermore, considerable discrepancies of N-*ras* mutation frequencies are reported between cultured metastatic melanomas (22%) and noncultured primary melanomas (5%) (Albino et al. 1989). This could be explained by an increased tendency to metastasis of NMM compared to SSM and the fact that most cultures are established by cells originating from metastases. However, even within the NMM, activation of N-*ras* seems to be involved only in a subgroup (31%) (our own results)

2. In melanoma cell lines it has been demonstrated, by means of immunohistochemical analysis, that *ras* mutation seems to be associated with the early to intermediate differentiation stage of melanocytes (Albino et al. 1989, 1992).
3. Epidemiological studies have revealed a causal role of sunlight exposure in the occurrence of cutaneous malignant melanoma (Tucker 1988). Ultraviolet (UV) radiation, as a component of sunlight, is especially thought to be the primary cause of melanoma formation (Howell et al. 1984; Sober 1987; Roth et al. 1988).

In vitro, UVR-induced point mutations in the human N-*ras* gene, involving CC and TT doublets at codon 12 and predominantly codon 61, have been demonstrated by means of transfection studies (van der Lubbe et al. 1988). In addition, it was demonstrated by ligation-mediated PCR, that the *ras* genes of UV-irradiated human cells show a high frequency of the two major types of potential mutagenic DNA photoproducts (cyclobutane pyrimidine dimers and (6-4)pyrimidone photoproducts). At codon 61 of N-*ras* (GTT) cyclobutane dimers were exclusively detected (Törmänen and Pfeiffer 1992).

In vivo, N-*ras* genes in melanomas carrying a point mutation predominantly located at codon 61 were found to be associated with intermittently to continuously sun-exposed body sites (van't Veer et al. 1989; Schrier et al. 1991). This suggests an etiological link between UV radiation and the activation of *ras* genes by point mutations. According to our investigation, all melanomas showing an activated gene were located on the back or upper arm, which constitute intermittently sun-exposed body sites. However, intermittent sun exposure occasionally cause sunburns, which may represent a higher risk for melanoma formation than moderate continuous sun exposure (Nelemans et al. 1993).

The frequent CAA to CGA mutation and all rare mutation types (Table 3) could be explained by UV-induced pyrimidine dimers.

The most prevalent mutation in human malignant melanoma (N-*ras* codon 61 [CAA (Gln) to AAA (Lys) transversion]) can not be explained by UV-induced pyrimidine

dimers, since the first base of this codon at the sense as well as at the antisense strand is not part of a dipyrimidine side (Table 1).

A possible explanation for the generation of this mutation may be the formation of 8-oxo-deoxyguanosine in the codogene strand at the first base of codon 61 followed by a mispairing event with adenosine in the codon strand: there is now convincing evidence that the major base modification caused by photosenzitation-generated singlet oxygen (Basu-Modak and Tyrell 1993) is 7,8-dihydro-8-oxo-deoxyguanosine (Epe 1991), which exhibits a prevalent tendency to mispair with adenosine (Cheng et al 1992). It has been demonstrated that near-visible light and UVB in the presence of endogenous photosensitizers like proflavin, hematoporphyrin and riboflavin induce high levels of 8-oxo-deoxyguanosine in cellular and cell-free systems (Yamamoto et al. 1992; Beehler et al. 1992; Epe et al. 1993).

According to a recent study, using PCR technology and subsequent oligonucleotide hybridization, Ki-*ras* mutation is a frequent event in dysplastic nevi and malignant melanoma (Shukla et al. 1989). This finding is in contrast to the results of other studies:

Raybaud et al. (1988), Albino et al. (1989, 1991), van't Veer et al. (1989) and Schrier et al. (1992) investigated melanocytic lesions independently by the same technique for point mutations in Ha- Ki- and N-*ras*-oncogenes. None of these authors reported detection of point mutations in the Ki-*ras* gene. This is consistent with our findings, revealed by mismatch primer-mediated RFLP-PCR and subsequent sequencing.

In summary, our data together with results of other authors support the hypothesis that activation of the N-*ras* and Ha-*ras* genes in human melanomas is associated with a specific subgroup of melanoma (NMM), characterized by an early differentiation stage as a possible subtype of NMM.

In the future, further studies with a larger number of samples are necessary, to evaluate the specificity and significance of our observations.

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