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Papers

Analysis of Expressed N-*ras* Mutations in Human Melanoma Short-term Cell Lines with Allele Specific Restriction Analysis Induced by the Polymerase Chain Reaction

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Mutations in *ras* genes have been found in the DNA of numerous cancer types including melanomas, but the expression of these mutations in melanomas has not yet been addressed. We have used the polymerase chain reaction (PCR) and allele-specific restriction analysis (ASRA) to determine the frequency of expressed N-*ras* mutations on 25 short-term melanoma tissue culture samples. N-*ras* cDNA generated using reverse transcriptase from whole cells was used as the PCR template. 14 secondary melanoma cultures that varied in differentiation patterns were analysed. Only 2 were found to express N-*ras* mutations; in both, the mutation was localised to one of the first two positions of the 61st codon of N-*ras*. These tumour lines, KMI-M8412a and KMI-M8412b, were established from separate tumour deposits in the same patient. Codons 12 and 13 were found to be free of mutations in all of the lines studied. 8 primary melanomas and 3 unclassified skin lesions were also analysed and found free of N-*ras* mutations. These results suggest that N-*ras* may not play such an important role in melanoma tumorigenesis as is speculated by others.
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INTRODUCTION

THE ACTIVATION or mutation of cellular oncogenes plays an important role in tumour biology. The *ras* oncogene family, H-*ras*, K-*ras* and N-*ras* encodes 21kD GTPase proteins (p21) that are similar structurally, functionally and immunologically [1]. *In vitro* mutagenesis studies in NIH 3T3 cells have identified critical codons in the *ras* genes (12, 13, 59, 61 and 63), that once mutated result in the activation of the gene and the subsequent transformation of the cells [2, 3]. Mutations in *ras* genes have been found in the DNA of numerous cancer types including approximately 20% of melanomas studied (Table 1).

In this paper, we report the frequency of expression of mutated N-*ras* alleles in malignant melanomas. This was determined using the polymerase chain reaction (PCR) and allele-specific restriction analysis (ASRA) on 25 short-term tissue culture samples, including 14 secondary melanomas, 8 primary melanomas and 3 unclassified skin lesions. N-*ras* cDNA generated using reverse transcriptase from whole cells was used as the PCR template. ASRA is a PCR based technology [4] that identifies the presence of mutations in codons 12, 13 and 61 of the N-*ras* gene, through the use of a series of mismatched primers each of which introduces appropriately positioned base substitutions in N-*ras* to create a restriction site provided the adjacent sequence is normal. Resistance of the amplified product to digestion indicates the presence of a mutation in the original template. The primer sequences and the conditions used were identical to those described by Todd and Iland [4] (Table 2).

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MATERIALS AND METHODS

The melanoma short-term cell lines, initially established and characterised in the Kanematsu Laboratories [5] were maintained under standard conditions in Dulbecco's Modified Eagles Medium (DMEM)(Flow Laboratories, North Ryde, NSW, Australia), with 4 mmol/l L-glutamine (Flow Laboratories) and 10% fetal calf serum (FCS) (Cytosystems, NSW, Australia). Supernatants (10 μl aliquots) of boiled cell lysates prepared from snap-frozen cells stored at −70°C were added to a 90 μl PCR reaction mix with a final concentration of;

Table 1. ras *mutations in melanoma*

Reference	Frequency	Most common *ras* mutation	Method
8	10/41	N-*ras* (codon 61)	PCR/ODN
11	1/13	N-*ras* (codon 61)	NIH 3T3
12	10/40	77% in K-*ras* (codon 12)	PCR/ODN
9	7/37	N-*ras*	ODN

ODN oligodeoxynucleotide hybridisation, PCR polymerase chain reaction.

Table 2. Sequence of N-ras primers used in PCR-based ASRA

Primer	Description	Sequence ± restriction site	Complement
E1M5a	5′ Mismatched primer for codon 12	5′ AACTGGTGGTGCCTGGAGCA *Eco*NI CCTNNNNNA*GG*	antisense
E1M5b	5′ Mismatched primer for codon 13	5′ AAACTGGTGGTGGTTCCAGCA *Pfl*MI CCANNNNNT*GG*	antisense
E2M5	5′ Mismatched primer for codon 61, positions 1 and 2	5′ GACATACTGGATACAGCTGG*C* *Msc*I TGGCCA	antisense
E1W5	5′ Wild type primer for exon 1	5′ TTCTTGCTGGTGTGAAAT	antisense
E2W5	5′ Wild type primer for exon 2	5′ ATGGTGAAACCTGTTTGTTG	antisense
E2M3	3′ Mismatched primer for codon 61, position 3	5′ CTCTCATGGCACTGTACAGTAC *Sca*I AGTAC*T*	sense
E1W3b	3′ Wild type primer for exon 1	5′ CCTCTATGGTGGGATCATATTCA	sense
E2W3	3′ Wild type primer for exon 2	5′ CCTGTCCTCATGTATTGGTC	sense

Underlined bases are not complementary to N-*ras* and result in the introduction of restriction sites. Bases in italics are the target nucleotides in the codon of interest.

50 mmol/l KCl, 10 mmol/l Tris (pH 8.3), 1.5 mmol/l $MgCl_2$, 0.01% (w/v) gelatin, each dNTP (dATP, dCTP, dGTP, dTTP) at 200 μmol/l, 50 pmol of both 3′(E2W3) and 5′(E1W5) wild type primers (Table 2), 20 units of RNasin (Promega, WI, USA) and 50 units of M-MuLV-reverse transcriptase (New England Biolabs, QLD, Australia). Reverse transcriptase reactions were carried out at 37°C for 45 min. After a 4 min, 94°C step to inactivate the reverse transcriptase, 3 units of Taq I DNA polymerase (Amplitaq, Perkin Elmer Cetus, USA), was added and 50 μl of mineral oil (Sigma, MO, USA) overlayed [6, 7]. The reactions were subjected to 35 cycles of amplification in a programmable heating block (Hybaid). A 12 μl aliquot of the PCR reaction was electrophoresed on a 4% Nu-sieve (FMC Bioproducts, ME, USA) agarose gel, stained with ethidium bromide and photographed. The primers and amplification conditions for N-*ras* were designed for two rounds of amplification (i.e. nested PCR); the first round spanned exons 1 and 2 and used cDNA not genomic DNA as the template (i.e. intron jumping). The 247 bp products of these reactions were then used as the templates for secondary codon-specific reactions which make up the ASRA technique [4].

RESULTS AND DISCUSSION

14 secondary melanoma cultures which varied in differentiation patterns were analysed. Only two samples were found to harbour N-*ras* mutations. These tumour lines, KMI-M8412a and KMI-M8412b, were established from separate tumour deposits in the same patient. In both samples the mutation was localised to one of the first two positions of the 61st codon of N-*ras*. Wild type transcripts were also identified in these samples indicating heterozygosity for the mutations (Fig. 1). Seven clones derived from KMI-M8412a by limiting dilution expressed the same pattern of mutant and wild type transcripts as the parent line. Codons 12 and 13 were found free of mutations in all of the lines studied. 8 primary melanomas and 3 unclassified skin lesions were also analysed and found free of N-*ras* mutations (Fig. 1). A high frequency of *ras* mutations has been identified in colon malignancies (50%) and pancreatic adenocarcinomas (80–90%), suggesting that *ras* activation is important in these cancers [1]. In contrast, the role of *ras* mutations in melanoma is less certain. Studies to date have identified *ras* gene mutations in 10–20% of malignant samples and/or cell lines, with the incidence in noncultured samples being somewhat lower (5–6%) [8]. Using the PCR-mediated ASRA technique on mRNA, we have demonstrated the expression of N-*ras* codon 61 mutations in 2/22 melanoma samples and 0/3 unclassified skin lesions. Codon 61 N-*ras* mutations have also been described in 7 patients with primary melanomas localising to frequently sun-exposed body regions, out of a total of 37 cases (19%) [9]. Because the mutations were primarily at or near dypyrimidine sites as found in codon 61, it was postulated that ultraviolet irradiation was active in tumour induction or progression. The only two N-*ras* mutations identified in this study were at codon 61 confirming the findings in other melanomas [8, 10–12], and were derived from tumours occurring in sun-exposed body sites.

The ASRA method is as sensitive as oligodeoxynucleotide hybridisation (ODN) or direct sequencing, with a level of sensitivity of 5% mutant sequences [4]. It also has several advantages over ODN or direct sequencing, in that it is quick, requires small numbers of cells (<100) and can detect the presence of a double mutation at adjacent codons which could be missed by ODN. Unlike previously published work demonstrating the presence of mutant alleles in genomic DNA, in this study we have been able to demonstrate that cells are expressing

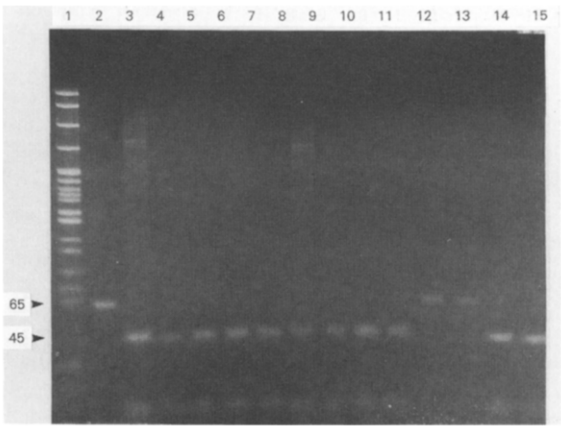

Fig. 1. Analysis of total RNA isolated from human short term melanoma cell lines for the presence of N-*ras* mutations in codon 61. Lane 2 contains a 65 bp amplification product of exon 2 which constitutes the secondary PCR reaction with mismatched primers targeting codon 61 (1st and 2nd codon positions). Lanes 3 to 15 contain 12 melanoma samples analysed by ASRA. Digestion resistant products of the secondary PCR reactions can be seen in lanes 12 and 13. Lane 1 is pBR322 digested with *Msp*I.

both mutant and wild type alleles. An algorithm based on the ASRA technique to determine the proportion of mutant to wild type sequences is currently being evaluated (Iland, H., unpublished results).

In conclusion, the low frequency of *ras* mutations expressed in melanoma suggests that they do not play a major role in this disease but may reflect genetic instability of the *ras* gene or of the genome in general. Our results do support the correlation between exposure to sunlight and N-*ras* codon 61 mutations in cutaneous melanoma.

1. Bos JL. Ras oncogenes in human cancer: a review. *Cancer Res* 1989, **49**, 4682–4689.
2. Barbacid M. Ras genes. *Annu Rev Biochem* 1987, **56**, 779–827.
3. Bos JL. The ras gene family and human carcinogenesis. *Mutat Res* 1988, **195**, 255–271.
4. Todd AV, Iland HJ. Rapid screening of mutant N-*ras* alleles by analysis of PCR induced restriction sites: allele specific restriction analysis (ASRA). *Leukaemia Lymphoma* 1991, **3**, 293–300.
5. Vincent PC, Joyce RM, Croaker G, Young GAR, Milton GW, McCarthy WH. Semi-solid cultures of primary melanomas and their correlation with histopathological features. *Pathology* 1985, **17**, 360–364.
6. Ferre F, Garduno F. Preparation of crude cell extract suitable for amplification of RNA by the polymerase chain reaction. *N.A.R.* 1989, **17**, 2141.
7. Sherman DR, Geliebter J, Cross GAM. Rapid and simple amplification of a specific RNA sequence by the polymerase chain reaction. *TIG* 1989, **5**, 137.
8. Albino AP, Nanus DM, Mentle IR, *et al.* Analysis of ras oncogenes in malignant melanoma and precursor lesions: correlation of point mutations with differentiation phenotype. *Oncogene* 1989, **4**, 1363–1374.
9. Van't Veer LJ, Burgering BM, Versteeg R, *et al.* N-*ras* mutations in human cutaneous melanoma from sun-exposed body sites. *Mol Cell Biol* 1989, **9**, 3114–3116.
10. Padua RA, Barrass NC and Currie GA. Activation of N-*ras* in a human melanoma cell line. *Mol Cell Biol* 1985, **5**, 582–585.
11. Raybaud, F. Noguchi T, Marics I, *et al.* Detection of a low frequency of activated ras genes in human melanomas using a tumourigenicity assay. *Cancer Res* 1988, **48**, 950–953.
12. Shukla VK, Hughes DC, Hughes LE, McCormick F, Padua RA. Ras mutations in human melanotic lesions: K-ras activation is a frequent and early event in melanoma development. *Oncogene Res* 1989, **5**, 121–127.

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EGF Receptor Amplification and Expression in Human Brain Tumours

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Human epidermal growth factor receptor (EGFr) gene amplification, rearrangements and expression were studied in tumours of the human nervous system. EGFr gene amplification was studied in 46 brain tumours. Gene expression was analysed by northern blot in 37 tumours and binding of its protein to EGF in 27 tumours. The *EGFr* gene was simultaneously amplified (with arrangements in 12.5% of gliomas) and overexpressed in 53% (9/17) of malignant gliomas, but never in meningiomas. In five high grade gliomas, amplification was always associated with a high level of receptors. However, since high amounts of EGF receptors found in one glioma were not the result of gene amplification, several systems of deregulation in EGFr production may exist and could be located at translational and/or post-translational levels.
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INTRODUCTION

THE HUMAN epidermal growth factor receptor (EGFr) is a single-chain transmembrane glycoprotein of molecular weight 170 kDa. The receptor includes an extracellular ligand binding domain, a transmembrane sequence, and an intracellular portion which has tyrosine kinase activity [1]. EGFr is activated by binding to EGF or EGF-like factors such as transforming growth factor alpha (TGFα). The v-*erb*B oncogene [2] encodes a truncated form of EGFr, and the loss of the extracellular portion results in constitutive phosphorylation of the receptor [3].

The EGFr gene is amplified and overexpressed in various tumoural tissues including brain neoplasia and human epidermoid carcinoma cells [4–6]. High EGF receptor levels have been observed in human gliomas [7, 8], and EGFr gene amplification has been described in 40% of glioblastomas [9, 10]. Abnormalities of EGFr have been found in the human glioblastoma cell line SF268 [11] in which an amplified EGFr gene appears to encode an enzymatically inactive protein [12]. EGF and TGF-α are the main peptides which bind EGFr. It has been shown that they have proliferating potential [13, 14].

The purpose of this work was to study a possible implication of the EGFr gene in human nervous system tumours. Amplification, mutations, and expression of EGFr gene as well as

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