Mutational analysis of the *N-ras*, *p53*, *p16*^{INK4a}, *CDK4*, and *MC1R* genes in human congenital melanocytic naevi

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Abstract

Eighteen human congenital melanocytic naevi (CMN) from 17 patients were screened for activating point mutations in the oncogenes *N-ras* and *CDK4* and for sequence variants in the *MC1R* gene by combined RFLP-PCR/SSCP analysis. In addition, all lesions were screened for deletions and point mutations in the tumour suppressor genes *p53* and *p16*^{INK4a} (*CDKN2A*) by combined multiplex PCR/SSCP analysis. Positive screening data were specified by sequencing of the corresponding PCR product. Activating point mutations in the *N-ras* gene (nine CAA (Gln) to AAA (Lys) transversions and one CAA (Gln) to CGA (Arg) transition at codon 61) were detected at high frequency (56%). Furthermore, three missense mutations (V92M) and two silent mutations (CGA (Arg) to CGG (Arg), codon 213, exon 6) were found in the *MC1R* and *p53* genes, respectively. No mutations were found in *p16* or *CDK4*. The activated *N-ras* oncogene, which is also found in human cutaneous melanomas, may constitute a potential risk factor for melanoma formation within CMN.

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Human melanocytic naevi are divided into two main categories, acquired and congenital. Most humans are born without any visible naevi. Approximately 1% of newborns exhibit single or multiple visible naevi at birth (congenital melanocytic naevi, CMN), which can vary in size from being less than 5 mm to covering extended body areas (giant congenital naevi).[1] The cause of CMN formation is unknown and methods of prevention do not exist. Congenital melanocytic naevi are of clinical importance, because especially the larger ones frequently grow into malignant melanoma. Thus CMN can be regarded as a potential melanoma precursor. The risk of melanoma formation within a single giant CMN is approximately 6% over an entire lifetime.[2] In spite of the general capability of CMNs to transform into malignancy, molecular risk factors defined at the gene level are unknown so far. Therefore, as a first approach to detecting possible CMN associated melanoma risk factors, we have screened 18 resected congenital naevi for the presence of mutations in five genes, which have been reported to be associated with human cutaneous melanoma (*N-ras*, *p53*, *p16*, *CDK4*, and *MC1R*). Activation of the *N-ras* oncogene within CMN may constitute a risk factor for melanoma formation, because activating *N-ras* mutations were reported in a relatively high proportion of spontaneous melanomas (5-35%) by different authors.[3] In 1994, Carr and Mackie[4] first reported point mutations of the *N-ras* gene in congenital melanocytic naevi also.[4] A single further *N-ras* gene mutation was described in a congenital naevus analysed by Jafari *et al*.[5] Inactivation of the tumour suppressor genes (TSG) *p53* and *p16*^{INK4a6} may constitute a further risk factor for CMN based melanoma formation, but these genes have not been studied so far in congenital naevi. Both genes function as negative regulators in cell cycle control. The upregulated p53 wild type protein also participates as a cardinal control factor in cellular response to DNA damage by arresting cell cycle progression in the G1 phase, thereby allowing DNA repair to be accomplished before cell division.[7 8] *p53* and *p16*^{INK4a} (*CDKN2A*) exhibit the highest TSG mutation rate found so far in human cancers.[9 10] Mutations in these genes have also been described in human cutaneous melanomas[11 12] and both genes seem to have a prognostic value concerning malignant progression.[13 14] A recently discovered *CDK4* point mutation, which prevents the cell cycle inhibitory effect of *p16*^{INK4a}, was also found in sporadic and familial forms of human melanoma. These findings support the candidacy of mutated *CDK4* in CMN as a further risk factor for the development of melanomas originating from congenital naevi.[15]

Based on epidemiological data, intense sunlight exposure constitutes a well established risk factor for melanoma formation.[16 17] In particular, occasional heavy sun exposure with a history of blistering sunburn in childhood and youth, predominantly occurring in subjects with blonde or red hair, blue or grey eyes, and a fair complexion, has been associated with an increased risk of melanoma.[18 19] Therefore, a further aim of our study was to find out if CMN mutations could be associated with episodes of heavy sunlight exposure causing sunburn in alliance with light skin type or hair colour.

Recently, an association of certain protein sequence variants in the melanocyte stimulating hormone receptor gene (*MC1R*) with red hair, fair skin, and cutaneous melanoma has

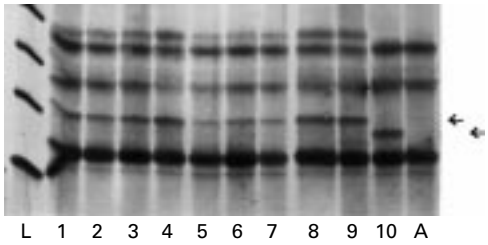

Figure 1 Silver stained SSCP gel comprising 10 congenital melanocytic naevi (sample numbers 1-10, table 1) harbouring dominant mutations in N-ras codon 61 along with an amnion sample as normal control (A) and a 100 bp ladder as size standard (L). The variant intensity of the mutation carrying bands (indicated by an arrow) indicate mutation carrying minor tissue subpopulations in certain CMN samples (particularly evident in samples 5 and 7). Congenital melanocytic naevus 10 exhibits an aberrant migrating band differing from all other samples (indicating a different point mutation).

been described,[20 21] so we also investigated *MC1R* variants in CMN.

Methods

DNA SAMPLES

DNA was extracted from snap frozen samples of 18 human congenital naevi from 17 patients taken from surgical resections by phenol/chloroform extraction as described previously.[5]

GENERAL PCR CONDITIONS

All PCR reactions were performed in a 100 µl volume with standardised buffer conditions[22] using a DNA Thermal Cycler (TC1, Perkin Elmer).

COMBINED RFLP-PCR/SSCP ANALYSIS OF *N-RAS*

In order to increase the screening efficiency for *N-ras* mutations (codons 12, 13, and 61) in cell subpopulations of CMN lesions, we coupled the traditional mismatch primer based RFLP-PCR analysis[5] with the more sensitive SSCP technique.[11] Very faint abnormal silver stained SSCP bands, which correspond to mutations in minor cell populations (fig 1), were excised and reamplified before subsequent sequencing (fig 2). Primer sequences for *N-ras* RFLP-PCR and a detailed protocol for combined RFLP-PCR/SSCP analysis and chemiluminescence sequencing have been described previously.[5 23]

COMBINED MULTIPLEX PCR/SSCP ANALYSIS OF *P53* AND $P16^{INK4a}$

Generally, tumours frequently harbour concomitant mutations in *p53* and $p16^{INK4a}$,[24 25] suggesting that the two genes operate via different pathways, each of which is important in suppressing malignant transformation. In contrast to *p53*, which is predominantly affected by missense mutations,[26–29] $p16^{INK4a}$, an inhibitor of *CDK4*, is most frequently inactivated by nonsense mutations and especially by homozygous deletions.[30–32] Using a combined multiplex PCR/SSCP analysis system, we screened all CMN lesions for homozygous deletions in $p16^{INK4a}$ as well as for loss of heterozygosity (LOH), microdeletions/insertions, and point mutations in $p16^{INK4a}$ (exons 1-3) and *p53* (exons 5-8). Primer sequences and a detailed protocol have been described previously.[33] Sequencing of SSCP detected *p53* exon 6 mutations of samples 3 and 4 (table 1) was accomplished by MWG-BIOTECH, Germany (sequencing data not shown) after reamplification of the mutation carrying band (enrichment of the mutated allele).

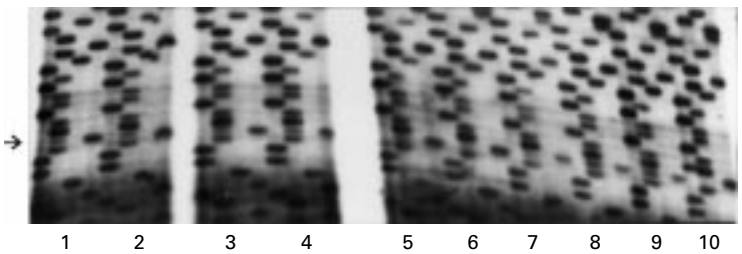

Figure 2 Sequencing (order of lanes: G, A, T, C) of the mutation carrying samples using mutant allele enrichment PCR product as template. The mutations in congenital melanocytic naevi 1 to 9 (CAA to AAA transversion) could clearly be determined (see arrow) after mutant allele enrichment PCR (reamplification of the gel excised mutation carrying bands, indicated by the arrow in fig 1). Congenital melanocytic naevus 10 harbours a CAA to CGA transition.

COMBINED RFLP-PCR/SSCP ANALYSIS OF *CDK4*

The *CDK4* gene was screened for the *p16* insensitive codon 24 Arg (CGT) to Cys (TGT) mutation, first described by Wölfel *et al*,[34] using combined RFLP-PCR/SSCP analysis. PCR reactions were performed according to standardised buffer conditions. DNA samples (500 ng) were denatured for five minutes at 94°C and incubated for 25 cycles for one minute at 94°C, one minute at 58°C, one minute at 72°C, with a final extension for five minutes at 72°C, using 300 ng of 1CDK4E2 (5' TTGGT GATAG GAGTC TGTGA 3') and 2CDK4E2 (5' TCCAG TCGCC TCAGT AAAGC 3'), respectively. *Sau*96I (G!GNC**C**) digestion of the 236 bp PCR product, resulting in 111 and 125 bp fragments, was performed according to the recommendations of the supplier (Promega). Combined RFLP-PCR/SSCP analysis was performed as for *N-ras* analysis.[23] SSCP analysis of codon 24 probably allows simultaneous screening of three further *CDK4* mutation sites, which were described more recently (codon 24, Arg CGT to His CAT, codon 22, Lys AAG to Gln CAG, and codon 41, Asn AAT to Ser AGT[15 35]).

COMBINED RFLP-PCR/SSCP ANALYSIS OF *MC1R*

PCR reactions were also performed with standardised buffer conditions. DNA samples (500 ng) were denatured for five minutes at 97°C, incubated for one cycle for two minutes at 94°C, two minutes at 60°C, three minutes at 72°C, and for 35 cycles for one minute at 94°C, one minute at 60°C, two minutes at 72°C, with a final extension for 10 minutes at 72°C, using 300 ng of MC1R1A (5' CTCCA CCCCC ACAGC CATCC 3') and MC1R1B (5' ATGTC AGCAC CTCCT TGAGC 3'), respectively (preamplification). For reamplification of the 899 bp PCR product with primer pairs MC1R2A (5' TGCAC TCACC CATGT ACTGC 3')/MC1R2B (5' GCACC GGCCT CCAGC AGGAG 3') (109 bp PCR product, codon D84E and V92M mutation screening) and MC1R3A (5' TCGTC CTCTG CCCCG AGCAC 3')/MC1R3B (5' CTGTG GAAGG CGTAG ATGAG 3') (115 bp PCR product,

Table 1 N-ras, MC1R, and p53 mutations in human congenital naevi

Patient	*Gender*	*Age*	*Lesion (cm)*	*Lesion location*	*Skin type*	*Hair colour*	*Eye colour*	*N-ras*	*MC1R variant*	*p53*
1	M	4	3.3×1.5	Hip	ND	ND	ND	**A**AA	No	No
2(a)*	F	12	1.0×1.0	Upper leg	III	Brown	Green	**A**AA	No	No
2(b)			0.5×0.5	Breast				No	No	No
3	M	10	5.6×2.8	Upper leg	III	Blonde	ND	**A**AA	No	CG**G**(213)
4*	F	4	3.5×1.9	Hand	II	Blonde	Blue	**A**AA	V92M	CG**G**(213)
5	F	16	1.2×2.0	Upper leg	ND	ND	ND	**A**AA	No	No
6*	F	12	12.×3.5	Buttock	II	Dark brown	Dark brown	**A**AA	V92M	No
7	M	16	6.0×1.2	Lower leg	IV	Brown	ND	**A**AA	No	No
8*	F	5	6.5×2.9	Upper arm	II	Blonde	ND	**A**AA	No	No
9	F	13	7.3×2.8	Lower leg	IV	Dark brown	Blue	**A**AA	No	No
10	M	16	3.5×2.5	Shoulder	III	Dark blonde	Light brown	C**G**A	V92M	No
11*	M	24	3.0×3.0	Armpit	II	Dark blonde	Blue-grey	No	No	No
12	F	18	1.5×1.0	Upper leg	III	Brown	Blue-grey	No	No	No
13	M	11	2.7×1.9	Neck	IV	Light brown	ND	No	No	No
14*	F	13	4.5×1.0	Back	II	Brown	Blue-grey	No	No	No
15	M	5	5.5×1.7	Flank	III	Dark blonde	Green-grey	No	No	No
16	F	6	3.0×1.3	Flank	III	Dark brown	Blue-grey	No	No	No
17	M	69	7.5×4.0	Back	III	Dark brown	Blue-grey	No	No	No

ND=not determined. *Reported sunburn.

codon D294H mutation screening), the preamplification product was excised with a sterile scalpel and dissolved in 500 µl of distilled water overnight at 4°C; 10 µl of this solution was used as template under otherwise identical PCR conditions. *Ava*II (G!**GWC**C), *Mae*II (A!C**GT**), and *Taq*I (T!C**GA**) digestion for mutation screening in codons 84 (resulting in 44 and 65 bp fragments), 92 (resulting in 67 and 42 bp fragments), and 294 (resulting in 88 and 27 bp fragments), respectively, was performed according to the recommendations of the supplier (Boehringer, Mannheim). Combined RFLP-PCR/SSCP analysis was again performed as for *N-ras* analysis.[23]

Results

In 18 investigated CMN samples from 17 patients, mutation of the *N-ras* gene was detected in 10 cases (56%) (table 1, patients 1-10) by combined RFLP-PCR/SSCP analysis and sequencing (figs 1 and 2). No *N-ras* mutations were found in corresponding adjacent normal tissue (data not shown).

All *N-ras* mutations were located in codon 61: CAA (Gln) to **A**AA (**Lys**) transversion at codon 61 of the *N-ras* gene was found in nine CMN. CAA (Gln) to C**G**A (**Arg**) transition at codon 61 of the *N-ras* gene was found in one CMN. No activating point mutations could be detected in codons 12 and 13 of the *N-ras* gene (data not shown). No gene inactivating point mutations or deletions could be detected in *p53* (exons 5-8) or $p16^{INK4a}$ (exons 1-3) by combined multiplex PCR/SSCP analysis (data not shown). However, a CG**A**(Arg) to CG**G**(Arg) silent mutation of codon 213 in exon 6 of *p53* was found in two CMN samples (data not shown). No point mutations could be detected in *CDK4* by combined RFLP/SSCP analysis (data not shown). The V92**M** missense mutation of the *MC1R* gene was detected in three CMN samples by combined RFLP/SSCP analysis (table 1, patients 4, 6, and 10). These mutations could also be found in corresponding adjacent normal tissue, thereby constituting germline mutations (data not shown).

Discussion

To our knowledge, this is the first report of mutation screening of $p16^{INK4a}$, *p53*, and *CDK4* in human congenital naevi. Neither gene inactivating point mutations nor homozygous deletions could be detected in $p16^{INK4a}$ or *p53*. Also, no activating point mutations were found in *CDK4*. These preliminary findings showed that *p53*, *p16*, and *CDK4* mutations are probably not involved in CMN based melanoma formation.

Concerning *N-ras* and *CMN*, we found activating point mutations with an unprecedented high frequency. However, out of 18 investigated CMN, only 10 lesions harboured *N-ras* mutations. In addition, in the patient with two simultaneously investigated naevi, only one lesion had a point mutation (table 1, sample 2a). Consequently, *N-ras* mutations seem not to be an essential primary event in CMN formation. Therefore, the existence of mutation carrying CMN subpopulations (fig 1, samples 5 and 7) could be considered as a strong indication for a chronological secondary mutational event.

In this context, UV radiation resulting from heavy sunlight exposure may be the most probable aetiological agent. However, pyrimidine dimers or (6:4) photoproducts should be excluded as a possible cause of the predominant **C**AA to **A**AA codon 61 mutation in CMN, because the nucleotides involved in this specific transversion event at position 1 of codon 61 (in both strands) are not components of a dipyrimidine site (GGA**C**AAGAA). Interestingly, the formation of 8-oxoG in the codogene strand at the first base of codon 61, followed by a mispairing event with adenosine in the codon strand, could be a possible explanation for sunlight induced generation of the **C**AA (Gln)→**A**AA (Lys) mutation. Cellular photosensitisers such as flavins and porphyrins absorb UV radiation and visible light and can react directly with DNA (type I reaction) or indirectly with oxygen (type II reaction), thereby generating reactive oxygen species like superoxide, hydroxyl radicals, and, particularly, singlet oxygen.[36] Probably the most frequently generated base modification resulting from the reaction of DNA with hydroxyl radicals and especially singlet oxygen is 8-hydroxyguanine (8-oxoG).[37] 8-oxoG mispairs with adenine during DNA replication[38] and consequently

gives rise to G:**C**→T:**A** transversions in bacteria[39] and mammalian cells.[40]

Particularly in massively pigmented naevi, phaeomelanin could constitute a further important endogenous cellular photosensitiser. The first indications for the mutagenic properties of the yellow-red phaeomelanin came from Harsanyi *et al*.[41] Using the Salmonella typhimurium histidine reversion test of Ames, they showed that phaeomelanin becomes mutagenic after exposure to long wavelength UV light. Furthermore, it was reported that phaeomelanin from human red hair produces considerably more cellular damage in Ehrlich ascites carcinoma cells when subjected to radiation of wavelength 320-700 nm than eumelanin from human black hair.[42] Using an in vitro system mimicking melanosomes (soy bean phosphatidylcholine liposomes), it was recently shown that phaeomelanin and its precursor intermediate 5-S-cysteinyldopa became pro-oxidant in the presence of ferric iron upon exposure to ultraviolet radiation, thereby generating superoxide radicals and singlet oxygen. This phenomenon was not observed with eumelanin and its precursor intermediate 5-6-dihydroxyindole.[43] Because melanosomes are known to possess several metal ions including Fe^{2+}, Cu^{2+}, and Zn^{2+}, superoxide radicals and singlet oxygen most probably are also generated in phaeomelanin containing melanosomes on sunlight exposure.

Interestingly, the **C**AA (Gln)→**A**AA (Lys) *N-ras* mutation also seems to be the predominant *ras* mutation in malignant melanomas.[5] Unfortunately, information concerning the skin type is not available. In the congenital naevi investigated by our group, we found **C**AA to **A**AA *N-ras* mutations in samples originating from patients with both lighter and darker colouration (skin types II, III, and IV, table 1). Our data could partly be explained by the fact that even people with darker skin types can exhibit variable amounts of phaeomelanin[44] and that ellipsoidal-lamellar eumelanosomes and spherical phaeomelanosomes can coexist in the same human melanocytes.[45] To evaluate our hypothesis, that a high frequency of *N-ras* **C**AA to **A**AA transversions in human congenital naevi might be associated with a high phaeomelanin/low eumelanin concentration in the respective melanocytes, further studies with a larger number of samples are needed. The best approach to this would be a comparative investigation of congenital naevi from black and red haired subjects, respectively. On one hand, the highest concentrations of epidermal eumelanin is found in people with dark hair. On the other hand, red haired people exhibit extremely high epidermal phaeomelanin/eumelanin ratios.[44 46]

In three samples, we detected the val 92 to met (V92M) mutation at the melanocortin-1 receptor (*MC1R*) in a heterozygous state. The mutation is thought to alter the alpha helix structure of the second transmembrane domain of *MC1R*. Valverde *et al*[20] reported an association of this *MC1R* variant with light and dark red hair and a tendency to sunburn. The polymorphism was also reported in 6.6% of subjects studied, predominantly those with blue eyes and blonde hair.[47] Xu *et al*[48] found the *V92M* variant in seven of 11 cases of skin type I (always burns, never tans).[48] Furthermore, they found a fivefold lowered binding affinity of *V92M-MC1R* to melanocortin-1 (melanocyte stimulating hormone, MSH) in COS-1 cells. In mammals, the relative amounts of eumelanin (black pigment) and phaeomelanin (red pigment) is regulated by the action of alpha-MSH on its receptor, the higher the affinity of alpha-MSH to its receptor, the greater the eumelanin level. It is interesting to note that we also found the polymorphism in two patients with blonde hair (in the other patient the hair colour was dark brown). These three patients also harboured *N-ras* mutations and two of them reported sunburn (table 1).

Another interesting aspect is comparing the mutation frequencies in $p16^{INK4a}$/*p53* and *N-ras* in congenital and dysplastic naevi, respectively. The absence of $p16^{INK4a}$ and *p53* mutations seems to be characteristic of congenital naevi (present study), whereas quite the opposite seems to be the case for dysplastic naevi. In a recent paper, 12 microdissected sporadic dysplastic naevi were screened for genetic alterations in $p16^{INK4a}$ and *p53*.[49] The authors found a hemizygous deletion at $p16^{INK4a}$ and *p53* in nine of 12 (75%) and six of 10 (60%) informative cases, respectively. Furthermore, four point mutations in $p16^{INK4a}$ and three point mutations in *p53* were detected.

Concerning *N-ras*, an extraordinarily high mutation frequency seems to be characteristic of congenital naevi, whereas absence of *ras* mutations seems to be characteristic of dysplastic naevi. We found *N-ras* mutations in CMN with an unprecedented high frequency. Furthermore, Carr *et al*[4] detected 12 point mutations in 43 congenital naevi (28%) by a dot blotting/oligo probing technique (6 × C**G**A, 5 × **A**AA, 1 × C**T**A).[4] Just as with our data, all mutations were exclusively located in codon 61, whereas no mutations could be detected in codons 12 and 13. In this context, the question arises of whether an aetiologically based connection may exist between congenital naevi and nodular malignant melanoma (NMM) because an association of NMM and *N-ras* mutations has been reported.[5 50] However, altogether 49 dysplastic naevi were investigated by three groups for point mutations in *N-ras* at codons 12, 13, and 61, but none was detected.[4 5 51]

This is the first report describing the striking differences in the spectrum of genes involved in congenital melanocytic naevi and dysplastic naevi. Further studies with a larger number of samples are necessary to evaluate the specificity and significance of these differences. By using microdissection techniques, congenital melanocytic naevi and dysplastic naevi together with primary melanomas originating from these lesions should be investigated, in order to gain information regarding the initial mutational steps involved in the formation of human cutaneous melanoma and its different subtypes (for example, nodular malignant melanoma, superficial spreading malignant melanoma). Furthermore, as an additional

approach, clearly defined cardinal skin types should be screened for mutations in selected genes, in order to find out if there really exists an association between phaeomelanin content of cells and types of mutations.

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